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Organotypic mouse brain slices: low-cost "ring-inserts" to study cholinergic and dopaminergic neurons with live cell imaging with an emphasis on calcium imaging

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Highlights:

- We developed a novel low-cost "ring-insert" to culture organotypic brain slices.
- Cholinergic and dopaminergic neurons survive on "ring-inserts."
- New nerve fibers grow along microcontact prints loaded with growth factors.
- These "ring-inserts" are useful for live-cell imaging of brain slices.
- Calcium imaging can be performed using Rhod-4 or Fluo-4 on brain slices.

Abstract: Organotypic brain slices preserve the complex 3D cellular structure and are the most potent *ex vivo* cultures, similar to *in vivo* conditions. However, their use is limited because of expensive membrane inserts and because the slices cannot be used for live-cell imaging. This study aimed to develop a low-cost and easy "ring-insert" to culture brain slices that can also be used for live-cell imaging. In this study, we created a "ring-insert" by gluing a low-cost (roll) membrane on plastic rings. These "ring-inserts" can be cultured with organotypic coronal mouse brain slices (150 µm) on top with the well-established interface method. In addition, these "ring-inserts" can be easily inverted and visualized under an inverse fluorescence microscope for live-cell imaging. We provide evidence that cholinergic (septum) and dopaminergic (mesencephalon) neurons survive on the "ring-inserts" for at least two weeks. Furthermore, we microcontact printed nerve growth factor and glial cell line–derived neurotrophic factor and visualized the outgrowth of cholinergic and dopaminergic neurons. Their activity was tested using fluorescent cell tracking and calcium imaging with Rhod-4/Fluo-4 after depolarization and calcium stimulation. In summary, a semipermeable 0.4-µm-pore membrane (Biopore Membrane BGCM0010, Merck Millipore) was glued onto 26 mm silicone O-rings by using Sylgard 184 polydimethylsiloxane prepolymer (Sylgard 184 Silicone Elastomer Kit, Dow Europe, Germany). Organotypic brain slices (150 µm) from postnatal day 10 mice were placed on top of these rings and cultured with the respective media underneath. The membrane has a hydrophilic polytetrafluoroethylene surface and becomes transparent when wet, thus allowing for the visualization of brain slices under a fluorescence microscope. The "ring-insert" represents a low-cost and easy model for culturing organotypic brain slices and allows for subsequent live-cell imaging.

Keywords: organotypic brain slices; “ring-insert”; cholinergic; dopaminergic; nerve fiber growth; live-cell imaging; microcontact printing

1. Introduction

Organotypic brain slice cultures bridge the gap between *in vitro* cell cultures and *in vivo* animal experiments [1]. In contrast to homogeneous single-cell cultures, the complex 3D architecture of the brain is preserved in organotypic brain slice cultures, thus allowing for the simulation of more *in vivo*-like situations [1–3]. Given that the main architecture of the cells is preserved, it allows for the *in vitro* investigation of cellular and molecular processes in the brain areas of interest. Gähwiler *et al.* were the first to successfully culture organotypic brain slices by using the roller-tube technique [4]. This technique was modified to maintain organotypic brain slices in cultures on semipermeable membranes [3]. We use the “chopper” technique and the “vibratome” technique in our lab, and we have extensively studied cholinergic neurons of the basal nucleus of Meynert (nBM), dopaminergic neurons, or serotonergic neurons (see review 1). A major benefit of organotypic brain slice cultures is the opportunity to perform co-culture experiments, which allows for the studying of two or more related brain areas [1,5], thus enabling the investigation of dopaminergic neurons with respect to Parkinson’s disease [6]. Similarly, we demonstrated the survival of cholinergic neurons in the nBM, thus allowing the exploration of the mechanisms underlying Alzheimer ́s disease [7]. Organotypic brain slice cultures are usually prepared from brains harvested on postnatal days 8–10 because tissue and cell survival remain high during this timeframe (see review 1). When the donor animal is older (> 14 days postnatal), tissue and cell death will occur more frequently in the culture. The brains of donor animals exhibit looser texture and morphology at younger ages. In addition, organotypic brain slices contribute to the reduce–refine–replace rule (3Rs) and markedly reduce the number of animal experiments.

Technically, the use of organotypic brain slices allows for a number of experimental strategies: studying the mechanisms of neurodegeneration in models of Parkinson’s or Alzheimer’s disease; studying the pathological cascades caused by beta-amyloid, tau, or alpha-synuclein; exploration of vascular angiogenic effects; or loading of molecules of interest using materials prepared using microcontact printing (µCP) to enable the establishment of a “brain-on-a-chip” [8]. Recently, we optimized the model to visualize glial fibrillary acidic protein–positive astroglia and laminin (LAM)–positive vessels in brain slices by using live-cell fluorescence imaging [9]. Organotypic brain slices have several disadvantages. First, this technique is highly complex and requires months of experience to prepare excellent slices. Second, membrane inserts (PICM03050, Merck Millipore) are extremely expensive, and the company holds a monopoly on these inserts, which can result in restrictions in delivery. Thus far, we have also tried inserts from other companies but have failed to achieve comparable slice quality. Third, the visualization of brain cells in multilayer organotypic slices using live-cell imaging for extended periods is difficult.

This study aimed to develop low-cost “ring-inserts” by gluing a membrane onto plastic rings. Brain slices (cholinergic and dopaminergic) will be cultured on these “ring-inserts,” and the outgrowth of neurons along lanes produced by µCP will be visualized. Second, live-cell imaging on these “ring-inserts” will show the activity of new nerve fibers and calcium imaging.

2. Methods

2.1. Preparation of "ring-inserts"

To prepare the "ring-inserts," we used silicone O-rings, with an outer diameter of 26 mm, inner diameter of 20 mm, and height of 3 mm (Weisser Berg, Amazon; one piece = EUR 0.654). These rings fit well into six-well plates. These silicone rings were "glued" onto a Biopore membrane roll (BGCM00010, Merck Millipore) by using a Sylgard 184 polydimethylsiloxane (PDMS) prepolymer (Sylgard 184 Silicone Elastomer Kit [GMID 01673921], Dow Europe, Germany) supplied as two components. The elastomer curing agent was carefully mixed with an elastomer base solution at a concentration of 1:10. The ring was then soaked in the PDMS mixture and placed on a large membrane roll soaked in aqua destillata (Figure 1A). The membranes glued to the PDMS were incubated overnight at 60 ℃, and then the "ring-inserts" were cut out (Figure 1B) and were ready for use in culture. We have not seen any damage to the rings and membranes caused by the 60 ℃ heating.

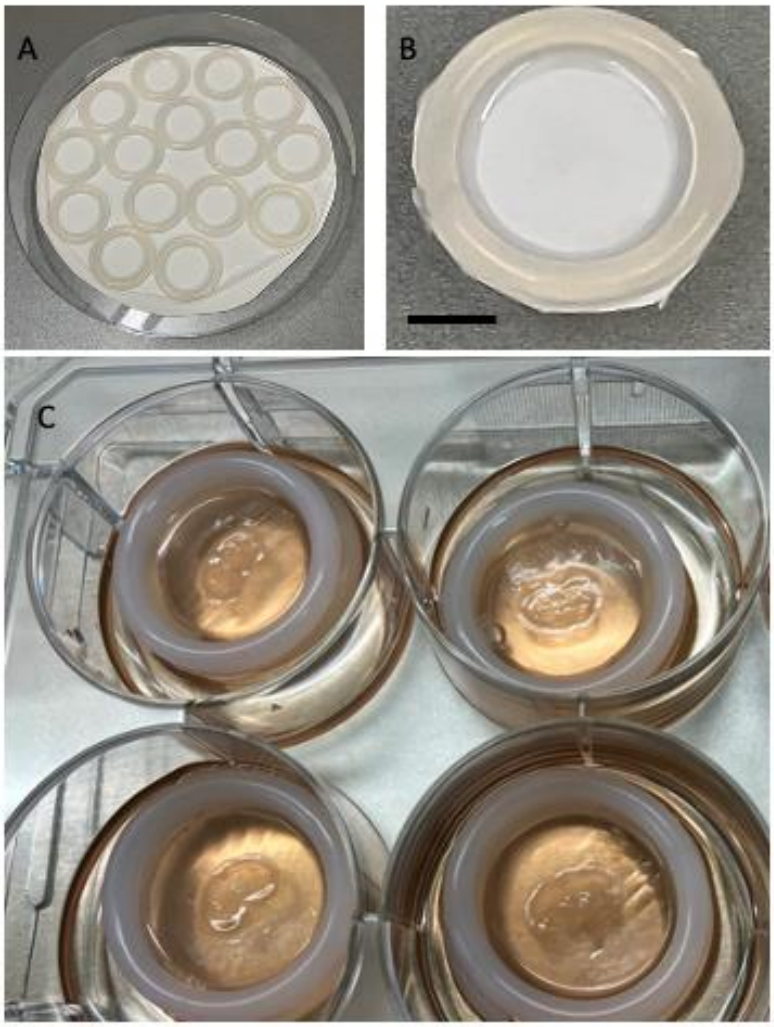

Figure 1. To prepare the "ring-inserts", silicone rings are glued onto a large piece of the membrane (BGCM00010) by using Sylgard184 **(A)**. These rings are cut out and sterilized for 2 × 10 min under ultraviolet light **(B)**. Brain slices can then be cultured in such ring-inserts on an additional extra ring positioned below **(C)**. Scale bar in **B** = 3.4 **(A)** and 0.85 cm **(B** and **C)**.

2.2. µCP of nerve growth factor (NGF) and glial cell line–derived neurotrophic factor (GDNF) on "ring-inserts"

The µCP was performed in a similar manner as that reported from our lab [9]. µCP uses an elastomer stamp, which adsorbs the "ink" solution and transfers it to a surface with a very high resolution (Figure 2). Approximately 100 µl of liquid collagen hydrogel ink solution (loaded with NGF or GDNF) was applied directly onto the micropatterned stamp. To evenly distribute the ink solution, a cover slip was gently placed on top. After 15 min of incubation at 37 ℃, the cover slip was removed and used to carefully strike off the remaining ink solution, once with and once against the lanes of the pattern. Excess solution on the borders of the pattern was removed using a filter paper without touching the printing surface or was left to air-dry for 1 min. As soon as the stamp was completely dry, the ink pattern was transferred to the "ring-insert" by applying pressure of 18 g for 60 min at room temperature. The position of the stamp was marked using four small dots on a permanent marker for the later arrangement of the slices. The weights and stamps were carefully removed from each corner. To verify the printing efficiency, a red fluorescent antibody was co-printed, and the lanes were marked. For the cell culture, the membranes were sterilized under ultraviolet light for 20 min. We did not observe any damage to the membrane and loaded proteins caused by ultraviolet treatments.

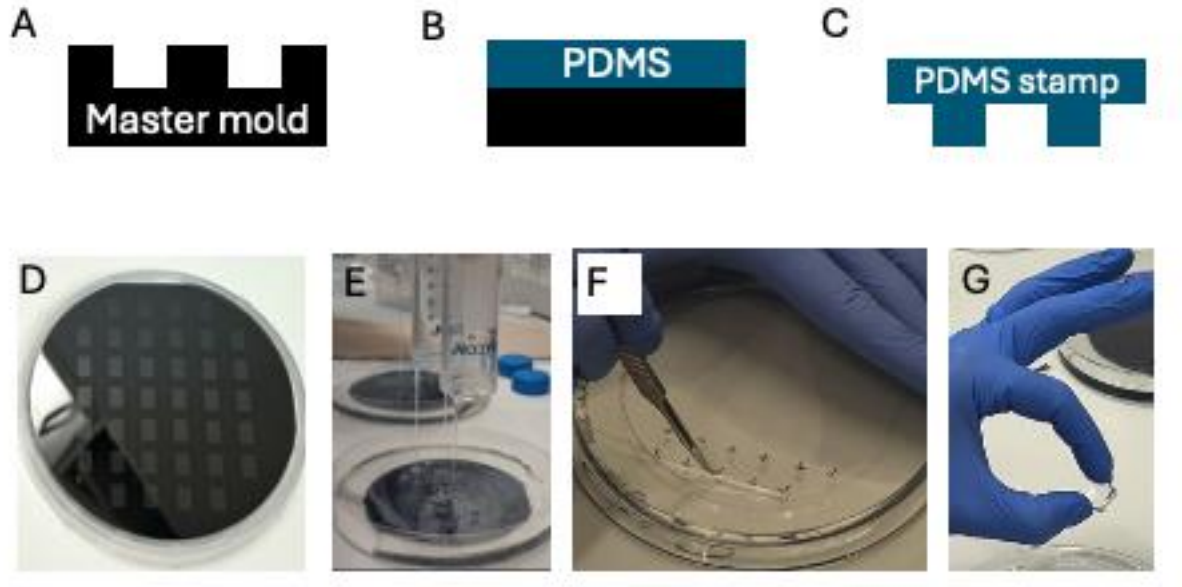

Figure 2. Polydimethylsiloxane stamps **(B** and **C**, blue**)** were generated from a master plate **(A** and **B**, black**)** with 30 µm lanes. The Sylgard compounds were poured onto the master **(D** and **E)** in a large petri dish and then incubated overnight at 60 ℃. The stamps were cut out with a scalpel **(F** and **G)**.

2.3. Organotypic brain slices cultured on "ring-inserts"

Organotypic brain slices were cultured on semipermeable membrane inserts as previously reported [7–9]. The brains were dissected under sterile conditions. After carefully removing the cerebellum, the brains were affixed onto the sample holder platform of a water-cooled vibratome (VT1000S, Leica, Germany)

with adhesive (Loctite 401 [11012044], Henkel, Germany231435) on their newly formed caudal surface. Coronal slices (150-µm thick) were cut in a sterile preparation medium and cooled down to approximately 5 ℃ (pH 7.2–7.3, autoclaved, 1 × minimum essential medium [11012044, Gibco, Thermo Fisher Scientific, USA], and 5.12 mM $NaHCO_3$ [106329, Merck Millipore]). Six slices from the frontal septal area (cholinergic) and six slices from the caudal ventral mesencephalon area (dopaminergic) were obtained from each mouse and positioned on cell culture inserts (Millicell, PICM03050, Merck Millipore). Cholinergic neurons were cultured on NGF µCP and dopaminergic neurons on GDNF µCP. To culture the ring inserts, a second silicone ring was used, and a 5 mm opening was cut out (to allow a medium change without bubbles). The silicone ring was sterilized and placed in a six-well plate. Then 1.7 ml medium was added and subsequently the "ring-insert" was positioned directly above the first ring. Slices were cultured for 2 weeks at 37 ℃ 5% CO_2 in a sterile slice culture medium: 1× MEM, 5.12 mM $NaHCO_3$, 31.5 mM glucose (49159, Merck Millipore), 2 mM glutamine (100289, Merck Millipore), 10% heat-inactivated horse serum (HS) (Gibco, 16050-122, Thermo Fisher Scientific, USA), 0.25 × Hanks' Balanced Salt Solution (Gibco, 24020091, Thermo Fisher Scientific, USA), 1 × antibiotic–antimitotic solution with pH 7.2 (A5955, Sigma-Aldrich, USA). To change the medium (twice a week), the medium was aspirated via an opening in the lower ring and replaced with a new medium. This enables it to be devoid of bubbles. The "ring-inserts" were compared to the standard original inserts from Merck Millipore (HTPP02500). For comparison, cholinergic neurons were cultured with 100 ng/mL NGF, and dopaminergic neurons were cultured with 100 ng/mL GDNF.

2.4. Viability of cells using 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI)

We did not observe any negative effects of the PDMS-prepared membranes or medium that could damage the material or slice quality. After two weeks, the cultured slices were stained with PI or for cellular homogeneity by using DAPI. The structures of the brain slices (flattening, damage, and brain region) were visualized using blue-fluorescent DAPI. The brain slices were fixed for 3 h in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), stained with the blue-fluorescent nuclear dye DAPI (1:10,000 dilution in Triton-PBS [T-PBS]) for 30 min, washed, and visualized in the blue channel. To measure cell viability, freshly cultured (non-fixed) slices were incubated with sterile-filtered slice medium containing the nuclear dye PI at a concentration of 2 µg/mL for 30 min at 37 ℃. Following incubation, the slices were washed twice with PBS, fixed with 4% paraformaldehyde for 3 h, and visualized using the red channel. Brain slices were treated with hydrogen peroxide (1 µl per 1 mL medium) overnight and used as positive controls.

2.5. Immunohistochemistry (microtubule-associated protein 2 [MAP2], glutamine synthetase [GluS], laminin, choline acetyltransferase [ChAT], tyrosine hydroxylase [TH], neurofilament [NF], p75)

Immunohistochemistry was performed as previously described under free-floating conditions [8,9]. This method allows the antibody to penetrate both sides during incubation, thereby enhancing staining sensitivity. First, the fixed brain slices were incubated in 0.1% T-PBS for 30 min at room temperature with gentle shaking. After incubation, the brain slices were washed for 3 × 3 min with PBS and subsequently blocked in 20% horse serum/0.2% bovine serum albumin (BSA)/T-PBS for 30 min under constant shaking. Following blocking, the brain slices were incubated in 0.2% BSA/T-PBS with primary

antibodies for 48 h at 4 ℃: for neurons with MAP2 (1:1000, SYSY 188002) or NF H (1:1000, SYSY nr. 171106, 1:1000), for astrocytes with GluS (1:500, abcam ab40873), for vessel with LAM (1:500, Sigma L9393), for cholinergic neurons with ChAT (1:750, Merck Millipore AB144P) or p75 neurotrophin receptor (1:1000, abcam ab529870) and for dopaminergic neurons with TH (1:1000, proteintech 25859-1-AP). After incubation, the brain slices were washed for 3 × 3 min with PBS and incubated with the corresponding green-fluorescent Alexa-488 secondary antibodies (1:400 in 0.2% BSA/T-PBS) for 1 h at room temperature while shaking. The secondary antibodies used were rabbit (MAP2, GluS, LAM, and TH), chicken (NF), and goat (ChAT) antibodies. The brain slices were counterstained with DAPI for 30 min, washed again with PBS, and directly visualized using a fluorescence microscope (Olympus BX61) connected to a Bresser camera (MikroCam PRO HDMI).

2.6. CellTracker live-cell imaging

To follow up on new nerve fibers by using live-cell imaging, brain slices were loaded with CellTracker. Slices underwent a 30 min wash with a serum-free medium at 37 ℃ and 5% CO_2. The brain slices were then loaded with CellTracker (Invitrogen C7025; 50 µg dissolved in 5 µl DMSO and 2 µl added per 1 ml medium) free-floating/inverted for 60 min at 37 ℃ and 5% CO_2. After loading, the membranes were washed once with serum-free medium and transferred back to their inserts, where they were incubated in serum-free medium for 2 h at 37 ℃ and 5% CO_2. The membranes were rinsed with 10 mM PBS and inverted under a microscope. The area of interest was focused on using a 10 × objective lens. Microscopic images were obtained on days 0, 2, 3, and 5. Sections were fixed and counterstained with NF and DAPI.

2.7. Calcium Rhod-4/Fluo-4 live-cell imaging

Calcium-sensitive dyes alter spectral properties upon binding to intracellular calcium ions (Ca^{2+}), thus providing insights into action potential dynamics. After a two-week culture period, the organotypic brain slices were loaded with Rhod-4 AM. Slices underwent a 30 min wash with a serum-free medium at 37 ℃ and 5% CO_2. Thereafter, the brain slices were loaded with the calcium-sensitive dye Rhod-4 AM (21121, AAT Bioquest, Pleasanton, USA) free-floating/inverted for 60 min at 37 ℃ and 5% CO_2. Rhod-4 was used at a concentration of 10 µM, with PowerLoad (P10020, Invitrogen, Thermo Fisher Scientific) at 1:100 and 2.5 mM probenecid (P36400, Invitrogen, Thermo Fisher Scientific) in serum-free medium. Following loading, the membranes were washed once with serum-free medium and transferred back to their inserts, where they were incubated in serum-free medium for 2 h at 37 ℃ and 5% CO_2 with 2.5 mM probenecid. The membranes were rinsed in 10 mM PBS and placed inverted under a microscope. The area of interest was then focused using a 10 × objective. The recording of a video was initiated, followed by the addition of 100 µL of isotonic 70 mM KCl solution on top of the membrane to induce depolarization; the effects were then observed for 5 min under the microscope (standard tetramethylrhodamine isothiocyanate filter set). Following depolarization, the organotypic brain slices were washed in PBS, fixed, and stained with NF and/or DAPI. In some experiments, equivalent amounts of Fluo-4-AM (Nr. F14201, Invitrogen) was used. For these experiments, calcium was stimulated with 70 mM KCl, 100 µM calcium ionophore A23187 (Nr. C7522, Sigma-Aldrich), 100 µM phorbol 12-myristate 13-acetate (PMA) (Nr. P8139, Sigma-Aldrich), or 100 µM ionomycin (Nr. 10634, Sigma-Aldrich). As a negative control for background signaling, the slices were stimulated with PBS only.

2.8. Data analysis and statistics

The number of TH+ and ChAT+ neurons from all six adjacent sections were counted under a microscope and averaged. The number of Rhod-4/Fluo-4+ stains was quantified manually using ImageJ software (version 1.54 g; National Institute of Health, USA) and PowerPoint (version 2503, Microsoft Corporation, USA). First, activated cells were determined in the videos; only cells with a clear change in fluorescence intensity and cells with nerve fibers were selected. DAPI and NF images were captured from the same section as the video at 10 × magnification and merged in PowerPoint. In parallel with the video analysis, cells that were activated and positive for DAPI and NF were marked. Finally, all neurons were counted with DAPI and NF images. The percentage of activated cells was calculated using the total number of cells. Only brain sections with clear outgrowths were used in the analysis. Optical density (OD) was analyzed using ImageJ by measuring the maximum fluorescence intensity and subtracting the background (zero value). The start and duration of the activation were visually determined. All values are expressed as mean ±standard error of the mean SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) with a subsequent Fisher's least significant difference posthoc test ($p < 0.05$ represents significance) or Student's t-test where appropriate.

3. Results

3.1. Quality of the brain slices on "ring-inserts"

The quality of the "ring-inserts" was compared to the standard membrane inserts from Merck Millipore. No differences in the slice quality were observed (see Supplementary Figure 1). To demonstrate the homogeneity of the cells, the slices were stained with the blue-fluorescent dye DAPI. All slices were flattened well and became transparent. The structure was very good, as exemplified at the hippocampal level, and there was no difference between the standard inserts and the "ring-inserts." In order to show viability, slices were stained with PI and no staining was seen on "ring-inserts" compared to standard inserts. As a positive control, the slices were incubated with peroxide and showed intense nuclear PI staining. To further characterize the quality of the slices, immunostaining was performed for neuronal MAP2, astroglial GluS, and vessel LAM. Fluorescence immunostainings did not show any differences when brain slices were cultured on standard Merck inserts compared with the "ring-inserts."

3.2. Cholinergic and dopaminergic neurons cultured on "ring-inserts"

Brain slices at the level of the septal area were cultured on "ring-inserts" and stained for ChAT. Several ChAT+ neurons were seen (Figure 3A), and quantitative analysis showed a slight decrease of neuronal survival on the "ring-inserts" (Figure 3B). Brain slices at the level of the ventral mesencephalon were cultured on "ring-inserts" and stained for TH. Several TH+ neurons were observed (Figure 3C), and quantitative analysis showed no difference between standard Merck inserts and "ring-inserts" (Figure 3D).

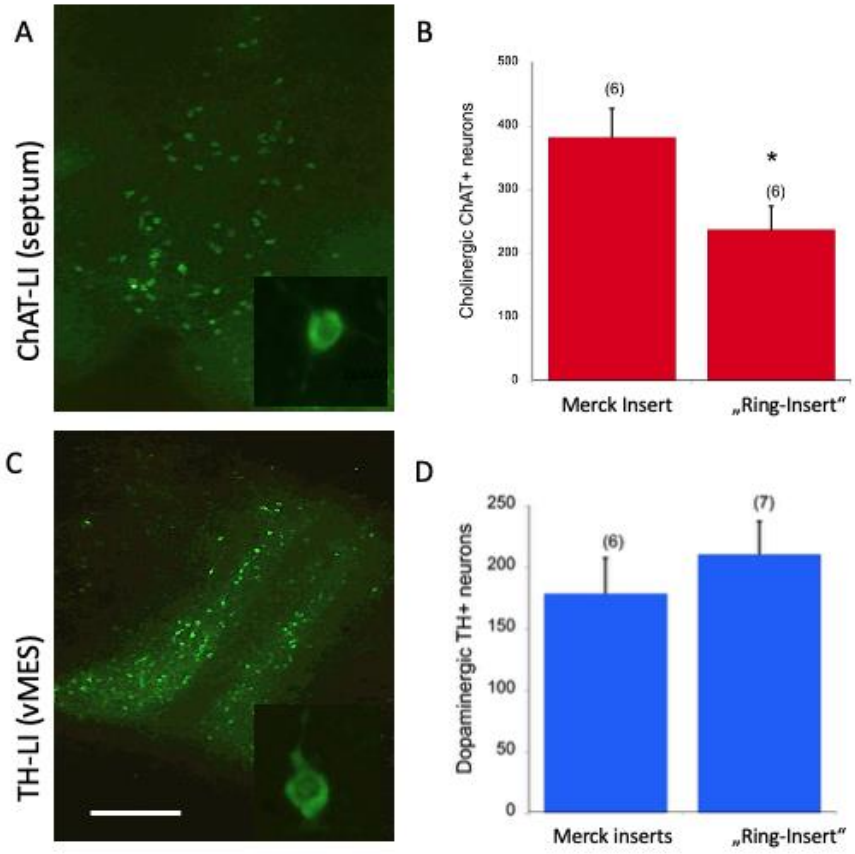

Figure 3. Cultures of cholinergic **(A)** and dopaminergic neurons **(C)** on "ring-inserts". Quantitative analysis shows a slight decrease of cholinergic neurons **(B)** but no difference of dopaminergic neurons **(D)** when cultured on "ring-inserts" compared with the standard Merck membrane inserts. Brain slices were incubated for two weeks with or without 100 ng/mL nerve growth factor or glial cell line–derived neurotrophic factor in a medium, fixed and stained for choline acetyltransferase (ChAT) or tyrosine hydroxylase (TH), and detected using green-fluorescent Alexa-488 antibodies. The number of ChAT+/TH+ cells was counted and statistically calculated using Student's t-test. Values are given as mean ± standard error of the mean, where the value in parenthesis indicates the number of used animals (*$p \leq 0.05$). Scale bar in C = 570 **(A** and **C)** and 57 µm **(**inserts in **A** and **C)**.

3.3. µCP and brain slices

Small lanes can be printed on the "ring-inserts" by using µCP and visualized as red fluorescent lanes (Figure 4A,B). Specificity was observed because there was no signal under the green channel (Figure 4C). Brain slices were labeled with blue-fluorescent DAPI (Figure 4E) and connected to the red lines (Figure 4D,F). Figure 4G shows a typical example of nerve fibers growing out of the slices, and Figure 4H shows the quantification of cholinergic and dopaminergic neurons.

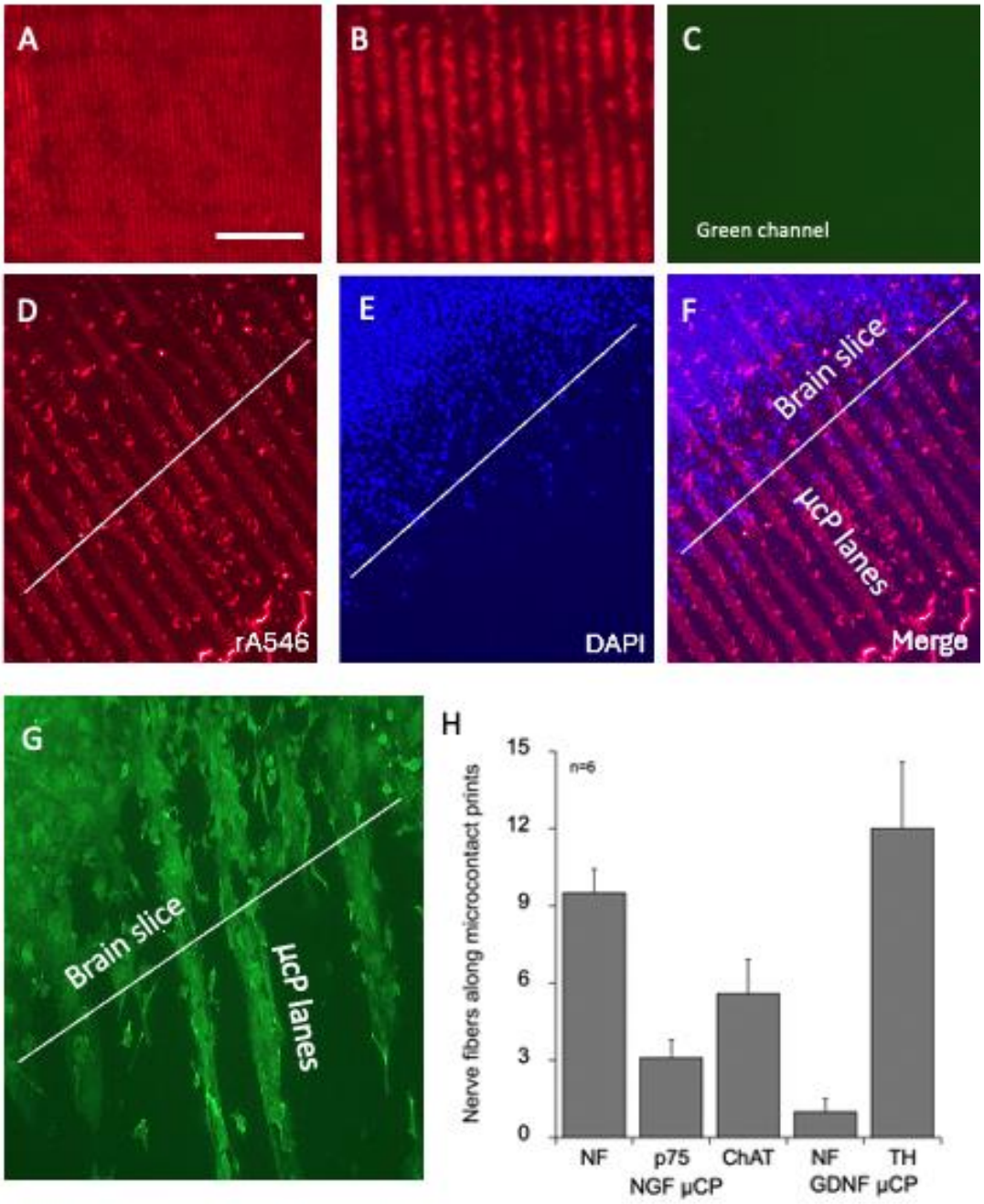

Figure 4. Microcontact printing and coupling to brain slices. A red fluorescent check antibody was microcontact printed on "ring-inserts" and shows many red lanes **(A** and **B)**, which is not evident in the green channel **(C)**. Organotypic brain slices (counterstained with blue-fluorescent nuclear 4', 6-diamidino-2-phenylindole; **E**) are connected to the red prints **(D)**, as shown in a merged picture **(F)**. Section G shows a typical example how nerve fibers grow along a microcontact printed lane; the white line shows the border between slice and outgrowth. Section H shows the quantification of the number of nerve fibers from one slice after two weeks in culture and after staining for neurofilament (NF), p75 neurotrophin receptor, choline acetyltransferase or tyrosine hydroxylase. Nerve fibers grew along the microcontact prints loaded with nerve growth factor (cholinergic neurons) or glial cell line–derived neurotophic factor (dopaminergic neurons). Values are mean ± standard error of the mean (n = 6 mice). Scale bar in **A** = 500 **(A)**, 170 **(B** and **C)**, 110 **(D–F)**, and 60 µm **(G)**.

3.4. Outgrowth of cholinergic and dopaminergic neurons along lanes

When cholinergic neurons were cultured on NGF microcontact prints, clear NF+ nerve fibers grew along the lanes (Figure 5A–C), which were positive for ChAT (Figure 5D–F) and p75 (Figure 5J–L). Similarly, dopaminergic neurons cultured on the GDNF microcontact prints grew along the lanes and were stained with TH (Figure 5G–I).

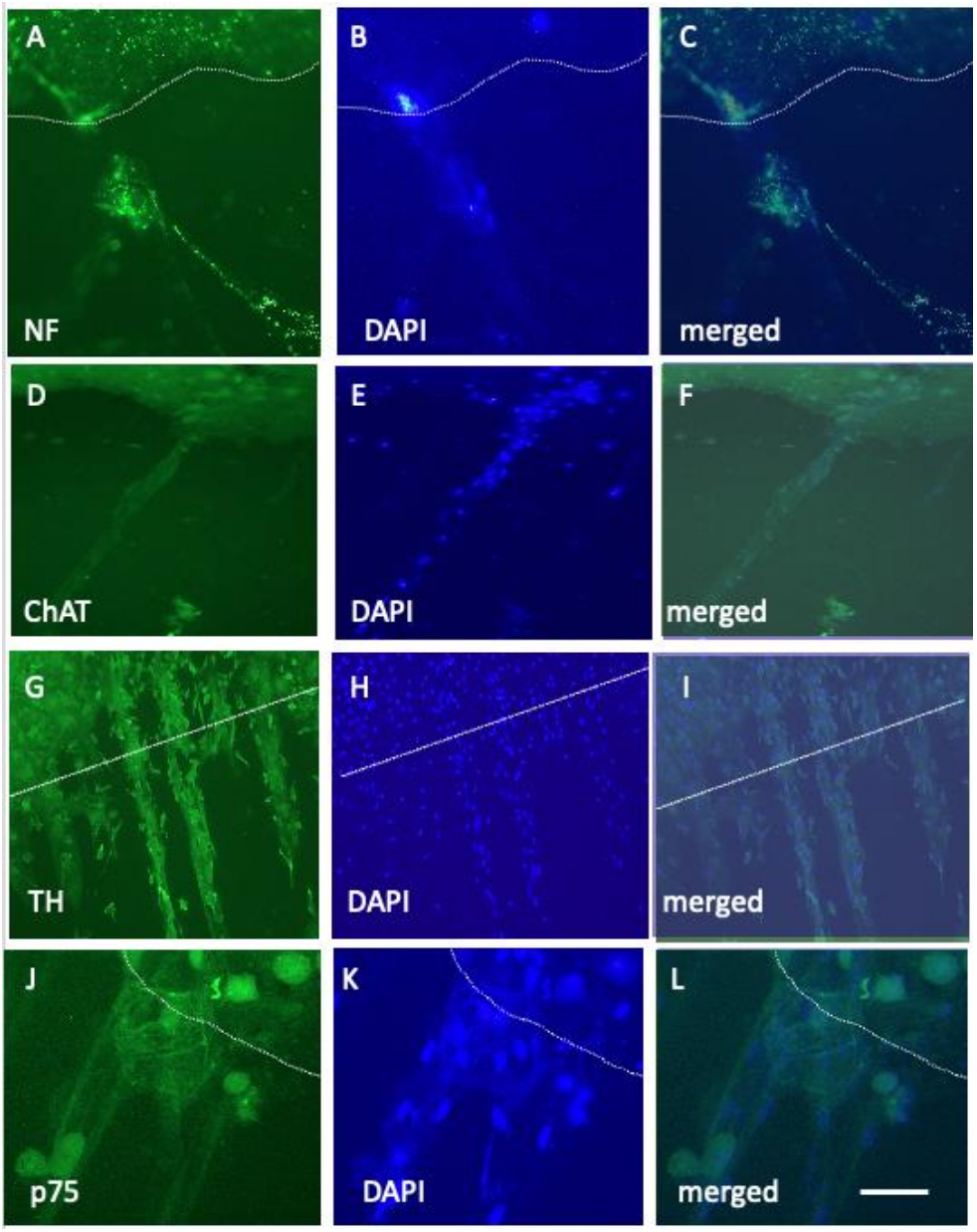

Figure 5. Outgrowth of neurons along lanes cultured on "ring-inserts". Cholinergic neurons grow along nerve growth factor microcontact prints **(A, D**, and **J)** and are stained with neurofilament **(A)**, choline acetyltransferase **(D)**, or p75 **(J)**. Dopaminergic neurons grow along glial cell line–derived neurotophic factor prints and are stained with tyrosine hydroxylase **(G)**. The slices were counterstained with blue-fluorescent nuclear 4',6-diamidino-2-phenylindole and showed the migration of cells along the prints **(B**, **E**, **H**, and **K)**. The merged pictures are shown in **C**, **F**, **I**, and **L**. Scale bar in L = 60 µm **(A–L)**.

3.5. CellTracker to follow up nerve fibers

Two-week-old slices were loaded with green-fluorescent CellTracker dye (Figure 6A**)** and visualized after one to five days (Figure 6B–D). CellTracker-labeled nerve fibers on the µCP were observed on the same day (Figure 6A). Incubation for over five days in the medium did not markedly change the fluorescence pattern (Figure 6B–D). Co-staining with NF (Figure 6E) and DAPI (Figure 6F) clearly showed that CellTracker labeled the nerve fibers.

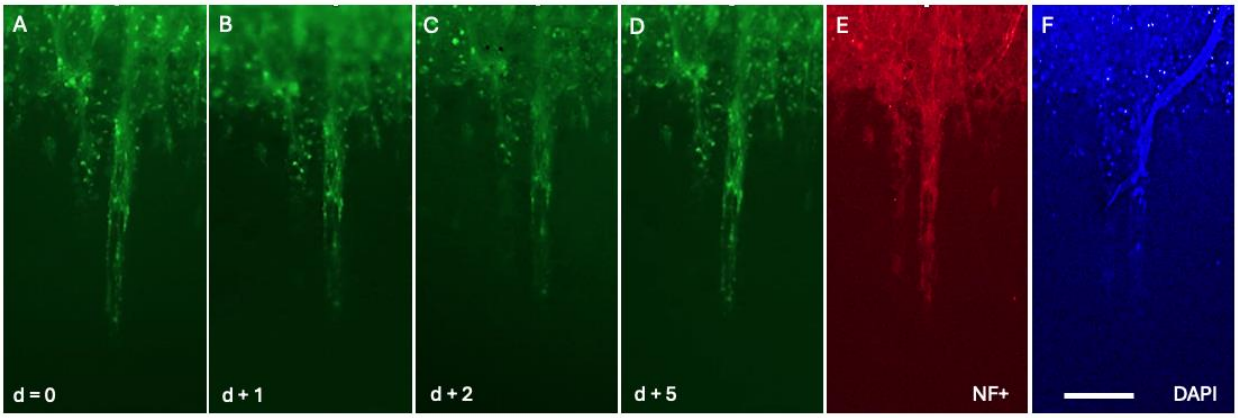

Figure 6. Labeling of new nerve fibers with green-fluorescent CellTracker. Two-week-old brain slices grown on nerve growth factor microcontact prints were loaded with CellTracker under living conditions. Nerve fibers could be visualized on the same day of loading with green fluorescent **(**day 0, **A)**. These nerve fibers remained stable up to five days in living brain slices **(**day 1, **B**; day 2, **C**; and day 5, **D)**. Co-staining with neurofilament **(E)** and blue-fluorescent nuclear 4',6-diamidino-2-phenylindole **(F)** showed the CellTracker-labeled nerve fibers. Scale bar in **F** = 95 µm (all).

3.6. Calcium imaging using Rhod-4 and Fluo-4

In two-week-old slices the non-fluorescent dye Rhod-4 was loaded, and the activation of newly outgrown nerve fibers was tested after depolarization with 70 mM KCl (Figure 7). At the start of the analysis, the fluorescence was low (Figure 7A), markedly increased after 70 s (Figure 7B), and returned to the background after 270 s (Figure 7C). The signal was clearly localized in the outgrown nerve fibers, as seen by NF and nuclear DAPI (Figure 7D–F).

Quantitative analysis of Rhod-4 showed that approximately 37 cells/mm^2 were activated by 70 mM KCl in the NGF-loaded slices and 34 cells/mm^2 were activated in the GDNF-loaded slices. They started after approximately 12–29 s and lasted approximately 156–182 s with an OD change of 22–25 (Table 1). Similar results were obtained using Fluo-4 (Table 2).

To test the specificity and selectivity of calcium imaging, brain slices were activated with the specific calcium dyes A23187, PMA, or ionomycin using Fluo-4. In comparison, the slices were also stimulated with 70 mM KCl depolarization, which was not different from that of the Rhod-4 dye (Tables 1 and 2). To stain for negative background signaling, the slices were stimulated with PBS only (Table 2). Calcium signaling was stimulated by A23187, PMA, and ionomycin, with PMA having the highest potency (Table 2).

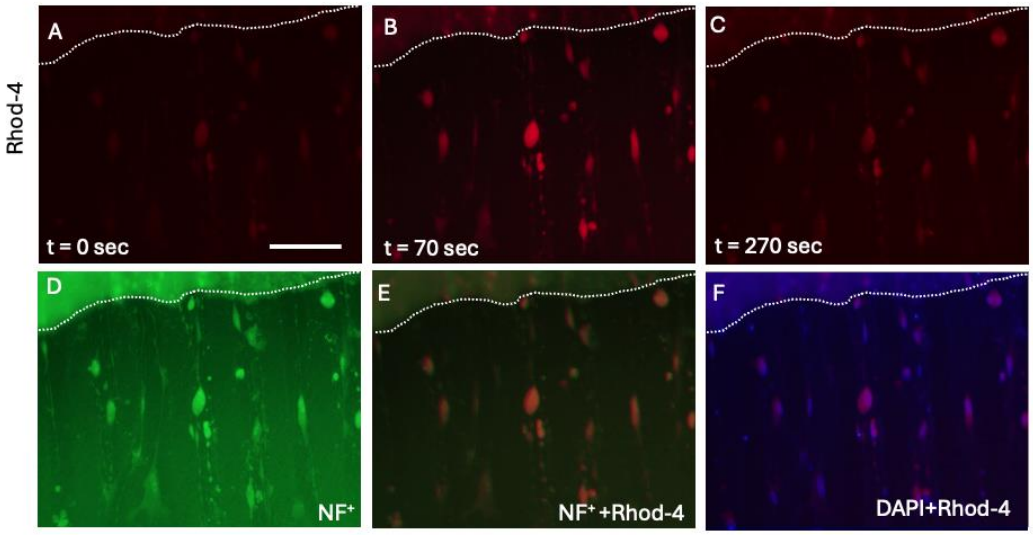

Figure 7. Calcium imaging with Rhod-4. Two-week-old slices were loaded with non-fluorescent Rhod-4, depolarized with 70 mM KCl, and analyzed for 5 min. Section A shows the start of depolarization, and sections B and C show the depolarization after 70 and 270 s, respectively. Strong red fluorescence was observed after 70 s. Slices were then fixed and stained for neurofilament **(D** and **E**, green fluorescent**)** or blue-fluorescent nuclear 4',6-diamidino-2-phenylindole **(F)**. Sections **E** and **F** show merged pictures. The white dotted line shows the border of the brain slice. Scale bar = 100 µm (all).

Table 1. Calcium imaging of new nerve fibers grown on microcontact prints using the calcium dye Rhod-4.

	µCP (NGF)	**µCP (GDNF)**
Activated cells/field	37 ± 5.2 (6)	34 ± 8.2 (8)
Start of activation [s]	29 ± 2.3 (6)	12 ± 5.8 (8)
Duration [s]	156 ± 21 (6)	182 ± 27 (8)
Optical density of activation	22 ± 0.7 (6)	25 ± 1.8 (8)

Brain slices were incubated for two weeks on materials prepared by microcontact printing (µCP) loaded with nerve growth factor (NGF) or glial cell line–derived neurotrophic factor (GDNF). The slices were loaded with the non-fluorescent dye Rhod-4 and then stimulated with 70 mM potassium chloride to induce depolarization. The activation of the cells was monitored in a field of 800 µm × 450 µm for 5 min, and the calcium-induced fluorescence was analyzed. Values are given as mean ± standard error of the mean; the values in parenthesis give the number of activated slices. The start and duration of activation is given in seconds. The optical density is the maximal stimulation minus the zero background. Statistical analysis was performed by Student's t-test.

Table 2. Calcium imaging of new nerve fibers grown on NGF microcontact prints using the calcium dye Fluo-4.

Activated cells/mm^2	**Start (s)**	**Duration (s)**	
PBS	17 ± 4.8 (6)	44 ± 9.9 (6)	132 ± 12.9 (6)
70 mM KCl	41 ± 4.9 (6) **	38 ± 7 (6)	153 ± 11.5 (6)
A23187 100 µM	41 ± 4.2 (6) **	39 ± 3 (6)	177 ± 4.6 (6) **
PMA 100 µM	58 ± 7.4 (6) ***	30 ± 2.2 (6)	149 ± 12.4 (6)
Ionomycin 100 µM	38 ± 4.6 (6) *	30 ± 5.1 (6)	154 ± 6.5 (6)

Brain slices were incubated for two weeks on materials prepared by microcontact printing loaded with nerve growth factor (NGF). The slices were then loaded with the non-fluorescent dye Fluo-4; stimulated with 100 µM A23187, 100 µM PMA, or 100 µM ionomycin; and compared with slices loaded with 70 mM KCl depolarization and background PBS only. The activation of the cells was monitored in a field of 800 µm × 450 µm for 5 min, and the calcium-induced fluorescence was analyzed. Values are given as mean ± standard error of the mean; the values in parenthesis indicate the number of activated slices. The number of activated cells is expressed as a percentage of total cells per mm2. The start and duration of activation is given in seconds. Statistical analysis was performed by one-way ANOVA with a subsequent Fisher's least significant difference posthoc test compared against PBS (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$). There was no statistical difference between the different treatments (KCl vs. A23187 vs. PMA or vs. Ionomycin).

4. Conclusion

We develop a novel "ring-insert" to culture organotypic brain slices and visualize them by live-cell imaging with an emphasis on calcium imaging. We show that these "ring-inserts" are low-cost and easy to prepare and that cholinergic and dopaminergic neurons survive for up to two weeks. In addition, nerve fibers grow along the microcontact prints and are viable and functionally active when imaged using calcium dyes.

4.1. Low-cost "ring-inserts" and quality

Organotypic brain slice cultures have been well established for more than 30 years in our laboratory, and many different brain areas and cells can be studied (see review 1). Usually, the slices are cultured on 0.4-µm-pore membrane (standard) insert from Merck Millipore by using the interface method, *i.e.*, slices are supported by a medium below and with O_2 and CO_2 from above. Under these conditions, the slices were flattened and became transparent, thus indicating survival. The membrane inserts of Millipore are very expensive, and one insert (PICM 03050) costs approximately EUR 15–20 (including taxes and overheads). Our "rings-inserts" are self-made and are cheaper (approximately EUR 2–3 per piece). For a typical experiment (200 slices), including controls and fallouts, approximately EUR 3,000 is needed. By contrast, 200 "ring-inserts" can be prepared for approximately EUR 500, thus indicating a cost efficiency of at least 2,500 euros for a small experiment of 200 slices. To guarantee good survival for the slices, it was necessary to increase the volume to 1.7 ml because the rings are thicker. When the medium needs to be changed, we used a second ring with a small entry to allow for the medium to be changed without causing air bubbles. It is essential that no air bubbles are below the slices and that no medium is above the slices. To compare the quality of the slices on "ring-inserts," we performed immunostainings for different brain cells and did not see a difference between the standard membrane inserts and our novel "ring-inserts" (see Supplementary Figure 1). Our data also showed that the slices on our "ring-inserts" were not damaged, survived well, and did not differ from standard membrane inserts.

4.2. Outgrowth of cholinergic nerve fibers along microcontact prints

We have extensive experience in culturing cholinergic neurons, particularly those from the nBM [7]. These neurons survived only when incubated with NGF [10–11]. In the present study, we investigated the

cholinergic neurons of the medial septum, which play crucial roles in memory, learning, and attention [12]. To stain these neurons, they were immunohistochemically labeled with antibodies against ChAT or the $p75^{NTR}$ receptor. Our data show that the cholinergic neurons survived well when cultured on the "ring-inserts"; however, their number was slightly lower than the standard Merck inserts. This could be caused by differences in the membrane surface hydrophilicity, nutrient diffusion efficiency, or, more likely, different medium application modes via the second ring or the slice heterogeneity. When the slices were connected to microcontact prints loaded with NGF, cholinergic nerve fibers grew along these microcontact prints. The survival and function of these cholinergic neurons are highly dependent on the availability of NGF, which binds primarily to the $p75^{NTR}$ and trkA receptors expressed on these neurons [13,14]. The loss of basal forebrain cholinergic neurons causes a significant decline in cortical and hippocampal acetylcholine levels and memory impairment in Alzheimer's disease [15–17].

4.3. Outgrowth of dopaminergic nerve fibers along microcontact prints

We have extensive experience in culturing dopaminergic neurons from the ventral mesencephalon, particularly from the substantia nigra [1]. These neurons only survive when incubated with GDNF, which is essential for their maintenance and axonal growth [18,19]. To stain these neurons, they were immunohistochemically labeled with antibodies against TH, a rate-limiting enzyme in dopamine synthesis. Dopaminergic neurons play a central role in the control of movements, and their degeneration results in Parkinson's disease [20–23]. Our data show that the dopaminergic neurons survive well when incubated on the "ring-inserts." When the slices were connected to microcontact prints loaded with GDNF, dopaminergic nerve fibers grew along these microcontact prints.

4.4. Cell tracker

Several fluorescent dyes are commercially available for labeling cells and follow-up using live-cell imaging, such as the commercially available Calcein-AM, Hoechst 3332, CellTracker, or Dil/Dio. In this study, we selected the green-fluorescent dye CellTracker Green CMFDA because of its low toxicity, strong signal intensity, and photostability, thus making it particularly suitable for long-term observations. By using our optimized protocol, we labeled brain slices within 60 min by using CellTracker and focused on the newly formed nerve fibers along the microcontact prints. Nearly all new nerve fibers were labeled with CellTracker immediately after loading. When we followed these slices for up to five days, fluorescent staining was still present, and no change in the fiber structure was observed. This was surprising because we expected the nerve fibers to grow further along the prints. It has been suggested that CellTracker is toxic, permanently fixes slices, or inhibits further growth. By using co-staining with neurofilaments, we clearly demonstrated that CellTracker labeled the nerve fibers. We can conclude that CellTracker is able to visualize neurons very rapidly but is not useful for the follow-up of live neurons. Our "ring-inserts" are well qualified to stain nerve fibers by using Cell Tracker and for follow-up.

4.5. Rhod-4/Fluo-4 live-cell imaging

Calcium is one of the most important ions for functional activity in neurons; it regulates intraneuronal processes and is important for synaptic function [24,25]. However, calcium imaging is complex and

difficult, and its use in brain slices is limited because the calcium imaging of multilayered tissues is technically challenging. This structure leads to light scattering and signal overlap, thus making the precise detection of calcium transients difficult. Calcium imaging has been successfully applied to organotypic acute brain slices (particularly in the hippocampus and neocortex) to study cellular and network dynamics in *ex vivo* preparations using Fluo-4 [26]. The calcium imaging of long-term brain slices is very difficult because brain slices are not in a single-cell layer. Advances in two-photon microscopy have made it possible to capture deeper tissue layers at a high resolution. Functional relationships can be analyzed more specifically [27]. The advantage of the present model lies in its ability to investigate newly formed outgrowth neurons along microcontact prints, which form a nearly single-cell layer. In the present study, we used two commercial potent calcium dyes, red-fluorescent Rhod-4 and green-fluorescent Fluo-4. The principle is that a non-fluorescent dye is loaded into neurons (with PowerLoad and probenecid) and then cleaved by intracellular esterases. Upon activation, calcium binds to the fluorophore, which is visualized as fluorescent light. In this study, we used depolarization with a high amount of potassium (70 mM KCl), which successfully activated neurons. Calcium-induced activation has been observed in both cholinergic and dopaminergic neurons. As a background control, only PBS was used, and low calcium stimulation was observed; however, depolarization with 70 mM KCl was significantly stronger. To better characterize calcium activation, cholinergic neurons were stimulated with three calcium-specific ionophores: PMA, ionomycin, and A23187.

PMA is known as a potent activator of protein kinase C (PKC), which is originally isolated from the plant *Croton tiglium*. It mimics the endogenous second messenger diacylglycerol, which is normally produced during cell stimulation. Compared with KCl depolarization, the effect of PMA on calcium response was approximately 17% stronger. This suggests that, in cholinergic neurons, PMA may be more effective than simple depolarization because it specifically activates PKC-dependent signaling pathways, whereas KCl relies on membrane depolarization and voltage-gated calcium channels.

Ionomycin is a calcium ionophore that transports calcium ions (Ca^{2+}) through biological membranes. Ionomycin specifically increases intracellular calcium levels independent of receptor- or channel-mediated mechanisms. Ionomycin induces a controlled and reproducible increase in intracellular calcium concentration. Therefore, it is a valuable tool for studying calcium-dependent cellular processes. In our experiments, the ionophor induced a calcium response but was less potent than PMA.

The drug A23187 is a calcium ionophore and is also known as calcimycin, which facilitates the transport of divalent cations, particularly Ca^{2+} and Mg^{2+}, across biological membranes. It increases intracellular calcium levels by transferring calcium into the cell along a concentration gradient. A23187 allows for a targeted and controlled increase in intracellular calcium levels independent of receptor-mediated signaling pathways. The ionophore A23187 activated the calcium response in cholinergic neurons; however, the cellular viability seemed to decrease, as shown by altered DAPI staining (data not shown). Therefore, we cannot exclude that the dose of A23187 was too high and induced toxic responses.

Taken together, all three drugs showed a potent response to calcium activation; however, to ensure reproducibility and minimize toxicity, a dose–response curve must be generated to identify the lowest effective non-toxic concentrations. In addition, these slices can be retested the next day or weeks later.

4.6. Limits of the study

This study has a few limitations. (1) Although the "ring-inserts" are easy to handle when culturing with brain slices, microscopy is tricky and challenging. The problem is that if the "ring-insert" dries out, the membrane becomes wavy; furthermore, the brain section is not flat, and wavy brain areas tend to become out of focus. (2) Given that specific regions of the brain (cholinergic and dopaminergic neurons) are examined, it is usually only possible to obtain six suitable slices per mouse brain. Particularly, it is technically challenging to prepare the frontal and caudal brain regions. (3) In a few stimulation experiments, some slices could not be used because no or only very small outgrowths of nerve fibers were found. The slices were then removed from the experiment. (4) Some slices could not be activated and were not included in the analysis because the morphologically differentiated neurites were damaged or did not express functional calcium channels. Alternatively, technical reasons, such as incomplete dye uptake or insufficient stimulation, could have also been the cause. (5) In some slices, nerve fiber outgrowth was observed as large nerve fiber bundles, which sometimes made it difficult to precisely assign the number of activated cells and cell nuclei. (6) We have not tested the cell viability after activation with calcium stimuli. However, we showed that 100 µM A23187 could be toxic to neurons. Additional viability assays, such as lactate dehydrogenase release and adenosine triphosphate detection, should be considered in future experiments to solve the problem of viability.

4.7. Future outlook and application scenarios

(1) The organotypic brain slice model is an excellent alternative to primary cell cultures and *in vivo* experimental slices and offers an alternative to animal research (3Rs). Although "organoids" are well established as another *ex vivo* alternative, "organoids" do not exhibit the complete regional brain architecture as postnatal brain slices [28]. Although we cannot completely replace animal experiments, many slices (50–100) can be prepared from 1 pup. The use of our novel cheaper "ring-insert model" may help achieve this aim. (2) All types of molecules, as well as cells or viruses, can be tested on brain slices and can be added either in the medium, directly on the slice, or locally using collagen scaffolds. This approach may offer a fast and effective method for developing novel pharmaceutical drugs [29]. (3) Live-cell imaging offers a new way to follow living brain cells by using µCP techniques. To date, we can follow up living brain cells for up to 7 h in a culture chamber connected to a microscope; we plan to connect delivery pumps, which may allow the follow-up of living cells over days or weeks. Many different applications [30–33], including spine imaging, neuronal circuits, myelin loss or calcium imaging, can be used with these slices. (4) In brain slices, vessels are expressed, and it will be challenging to connect brain slices to the blood–brain barrier. Endothelial cells, pericytes, and astrocytes can be cultured on one side of the membrane and may form a functional blood–brain barrier [34], where diffusion or active transport from one side to another can be studied. (5) Brain slices offer the advantage of studying brain disorders such as Parkinson's disease and Alzheimer's disease, and prion-like molecules can be added to study the spread and progression of these diseases. In this context, brain slices from transgenic mice [35,36] can be cultured. (6) This approach offers the opportunity to culture brain slices from adult animals [37,38]. Considering that most brain disorders in humans are due to old age, the application of mouse postnatal slices to human research is low. Culturing adult transgenic slices will be challenging in the future. (7) Finally, the slice model may facilitate the culturing of human adult

tissues [39,40] and the direct investigation of its effects in humans. Unfortunately, adult human cells only survive for a few days.

We demonstrate for the first time that our novel "ring-inserts" provide a potent and low-cost alternative compared with the expensive standard membrane inserts from Millipore. We showed that the slices remain viable and that both cholinergic and dopaminergic neurons can be studied using this setup. Growth factors (NGF and GDNF) can be printed onto these "ring-inserts" by using μCP, and cholinergic and dopaminergic nerve fibers grow along the NGF and GDNF prints, respectively. Finally, we showed that these newly formed nerve fibers are functionally active and can take up CellTracker. Furthermore, calcium activation can be explored. In conclusion, the "ring-inserts" are a low-cost method for culturing organotypic brain slices and can be used for the live-cell imaging of brain slices.

5. Supplementary data

The authors confirm that the supplementary data are available within this article.

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Author's contributions

Conceptualization, CH; methodology, CH and AG; investigation, CH, AG, PL, and JS; resources, CH; data curation, CH, AG, PL and JS; writing—original draft preparation, CH, PL, JS and AG; writing—review and editing, CH and AG; visualization, CH; supervision, CH; project administration, CH. All authors have read and agreed to the published version of the manuscript.

Conflicts of interests

The authors declare no conflicts of interest.

Ethical statement

All animal experiments were approved by the Austrian Ministry of Science and Research (66.011/0055-WF/V/3b/2017 and BMWF-66.011/0120-II/3b/2013) and conformed to the Austrian guidelines on animal welfare and experimentation. Our study using animals (mice) followed the ethical guidelines for sacrificing animals, and our animal work complied with international and national regulations. All experiments were performed according to the 3Rs rule of animal experiments. All our slice experiments are defined as "organ removal" and are not "animal experiments."

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