DACIT: Device for Axon - Cancer cell Interaction Testing in 2D and 3D

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Summary

In this protocol, we describe steps to design, fabricate and use the Device for Axon and Cancer cell Interaction Testing (DACIT) in 2D and in 3D. In the first section, we detail steps to generate the mask, the master and the smooth-on mold. Next, we describe the step-by-step protocol for fabricating the DACIT, loading sensory neurons and cancer cells in 2D or 3D. We compare axonogenesis using PC-12 cell line and primary embryonic or adult sensory neurons, demonstrating the superior neurite growth in primary cells. We demonstrate DACIT can be used to compartmentalize neuronal soma and axons and expose them to different conditions, or to form a temporary gradient of neurotransmitter. Finally, we show that DACIT can be used to measure spheroid invasion in 3D in the presence of axons.

Introduction

Cancer cell interactions with sensory nerves and neurons are generally understudied. Several technical reasons can explain this gap in knowledge. First, the soma (cell bodies) of the peripheral neurons are located in ganglions, in the vicinity of the spinal cord. In humans, this location can be >1 meter away from the tumor that the ganglion innervates. Hence, only axons, axon bundles (fascicles) and nerves will be in physical contact with tumor cells and providing tumor innervation. Mimicking this compartmentalization in experiments is important not only to maintain similarity to anatomy, but also to avoid experimental artifacts. For example, the fetal bovine serum (FBS) used for culturing most cancer cell lines has a high concentration of glutamate, which can cause neurotoxicity to neurons[5].

Second, neurons can be hard to maintain in culture and there are almost no physiologically relevant cell culture models available. Some studies use cell lines, such as PC12, which are better in modeling sympathetic neurons compared to sensory ones. Most studies, however, use primary culture to study peripheral neuron behavior. This approach limits the available number of neurons and generates an additional issue of the presence of non-neuronal cells, such as Schwann cells, which are difficult to eliminate and often affect neuronal behavior.

Several recent studies demonstrated a role of the peripheral nervous system in tumor growth and metastasis, including pancreatic, melanoma and breast cancer[1-4]. These observations generated an increased interest in understanding the molecular and cellular mechanisms that drive interactions between neurons and cancer cells, and generating appropriate technical solutions for such studies.

To provide compartmentalization between somas and axons, the Campenot chamber[6] was developed, which consisted of a collagen-coated Petri dish with scratches to guide axon growth. The introduction of the microfluidics to the Campenot chamber[7] divided the chamber into three compartments using a Teflon divider, with one compartment containing neuronal soma and two compartments containing axons. This approach allowed cellular compartmentalization and fluidic isolation and has been used to successfully perform several cellular and biochemical assays[7]. More recently, the progress of microfabrication and the inspiration of the original concept of the Campenot chamber triggered the development of a series of compartmentalized microfluidic devices to analyze anything from drug screening[8] to axonal regeneration[9, 10] in 2D cultures

The culture of cells in 3D in cancer research represents a physiologically relevant alternative to animal models, as it better recapitulates tumor cell behaviors in vivo such, including proliferation, morphology, and drug resistance[11, 12], and allows genetic and chemical manipulation and imaging. Here, we present a novel Device for Axon Cancer cell Interaction Testing in 2D and 3D (DACIT). DACIT allows monitoring of the interactions between axons and cancer cells in 2D or in 3D culture, using cancer cell spheroids embedded in 3D extracellular matrix. DACIT can be used to expose neuronal soma and axons to either same, or different media conditions, using media compartmentalization and gradient formation.

Before you begin

The protocol below describes the steps for fabricating a SU-8 DACIT master and a duplicate as a Smooth-On mold. Next, we explain step-by-step how to fabricate DACITs from the master

and how to embed neurons and cancer cells spheroids. In this protocol, we used either a primary culture of DRG neurons or the cell line PC12, but induced pluripotent stem cells differentiated to neurons or a primary culture of another type of neurons can also be used. As a cancer cell model, we used the 4T1 breast cancer cell line, but other cell lines from the breast or other cell types can be used.

Institutional permissions

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

Photomask design and ordering procedure.

DACIT consists of two wide channels (1.5 mm) connected by microgrooves which are 5 µm in width, preventing cells but allowing axons to cross the channels[10] To fabricate drastically different heights of 3 µm (microgrooves) and 500 µm (channels), we have separated the device design into two layers (**Figure 1**) and fabricated two masks, which were aligned during master production.

The DACIT design was generated utilizing LayoutEditor. Layers are incorporated to reach two different heights. **Figure 1A** illustrates the designed layers, showcasing microgrooves (blue, **Figure 1B**) and channels (red, **Figure 1C**). Alignment marks are included in both layers to facilitate precise alignment during fabrication. Go to the “Resource availability” section for GDS files.

To fabricate the designed DACIT, precision in printing the layers onto photomasks is paramount. The minimum distance between lines or objects in the design dictates the required precision in the printing process. The microgrooves layer necessitates the smallest feature of 5 µm, while the microchannel layer requires 300 µm precision.

Photomasks for each layer were ordered from different vendors. The microchannel layer photomask is sourced from CAD/Art Services, Inc., using transparency film as the substrate (https://www.outputcity.com/). The microgrooves layer photomask is obtained from Compugraphic, employing chrome mask technology (https://www.compugraphics-photomasks.com/). **Table 1** outlines the specifications for each order of photomasks, detailing precision and substrate material.

Mask-type	Vendor	Product	Material Type	Min Feature Size (µ)	CD Tolerance ± (µ)	Defect Density (µ)
Chrome mask	Compugraphic	Master 1X	5" x 5" x 0.090" QZ	5.0	0.5	5.0
Transparency film	CAD-Art Services	10,000 dpi mask	Transparency film	8.0	N/A	N/A

Table 1. Photo masks specifications

Preparation of the SU-8 master

Timing: [4 days]

Day 1

1. Preparation of DACIT SU-8 master (**Figure 2A-C**). For the best results, the protocol should be carried out in a clean room equipped for soft lithography.

a. Use the 10 cm silicon wafer to generate the master. Inspect the silicon wafer for any residue or imperfections. If the wafer has residue, rinse it first with acetone and then with isopropanol. Dry it with pressurized nitrogen gas.
b. Heat the wafer on a hot plate at 200°C for 5-15 min

2. Microgrooves preparation
   a. Place the wafer in the spin coating machine and add around 4 ml of SU-8 2005 to the wafer.
   b. Spin coat SU-8 2005 onto the wafer in two steps:
      i. Step 1: Speed of 500 rpm, and ramp of 100 rpm/s for 7-10 sec
      ii. Step 2: Speed of 3000 rpm with a ramp of 300 rpm/s for 30 sec
   c. To soft bake the SU-8, carefully transfer the SU-8 coated wafer to a hot plate set to 95°C and let it sit there for 2 min.
   d. In the mask alignment machine, align the wafer and the chrome mask so that the microgroove pattern is centered on the wafer.
   e. Expose the SU-8 to 90 mj/cm^2 UV light. The exposure time will depend on the power of the UV source.
   f. Bake the wafer post-exposure at 95°C for 2-3 min

3. Channels preparation
   a. Place a piece of tape on the edge of the wafer covering only the alignment marks and leaving some tape hanging over the edge. Fold the piece hanging over onto itself so that the adhesive part is covered (see troubleshooting section).
   b. Place the wafer with the microgrooves onto the spin coating machine and add ~5 ml of SU-8 2100 to the wafer
   c. Spin coat SU-8 2100 onto the wafer in two consecutive steps.
      i. First, for 10 sec at 500 rpm with a ramp of 100 rpm/s
      ii. Second, for 35 sec at 1500 rpm with a ramp of 200rpm/s
   d. Place the wafer on a hot plate set to 23°C and gradually increase the temperature to 55°C at a rate of 25°C/h. Leave the wafer on the hotplate overnight at 55°C

Day 2

   e. The following day, gently peel off the tape while the SU-8 is warm being careful not to remove more SU-8 than is on the tape.
      Critical: The SU-8 must be warm or you may not be able to cleanly remove the tape to reveal the alignment marks.
   f. Gradually cool the wafer and SU-8 to room temperature by decreasing the temperature from 55°C to 23°C at a rate of 25°C/h.
   g. Repeat steps 3b through 3f to create the second half of the channel layer.
      Critical: It is important to wait until the SU-8 from the previous layer is completely cool before adding the next layer of SU-8.

Day 3

   h. Align the transparency for the main channels with the alignment mark from the microgrooves layer using the mask aligning machine.
      Critical: Alignment must be done accurately to prevent the channels from covering the microgrooves.
   i. Expose the main channels and wells to UV light on the mask alignment machine.
   j. Bake the wafer by placing it on the hot plate set to 23°C and gradually increase the temperature to 55°C at a rate of 25°C /h.
   k. Bake the wafer overnight at 55°C to achieve maximum height.

Day 4

l. The next day, slowly cool the wafer by decreasing the temperature of the hotplate from 55°C to 23°C at a rate of 25°C/h.
m. Develop the SU-8 by placing the wafer in a glass dish and cover the wafer with SU-8 developer. Swirl the wafer in the development solution for 30 min to 1 h until the unexposed SU-8 is dissolved.
n. Hard bake the wafer at 200°C for 15-30 min.
o. Wash the finished SU-8 master with acetone followed by isopropanol and store it in a closed container to prevent dust and debris from falling on it.

Preparation of the Smooth-On mold

Timing: [24 h]

Smooth-on mold provides an efficient and cost-effective duplicate of the SU-8 master[13] (**Figure 2D**).

1. Create a PDMS master from the SU-8 master following the soft lithography portion of the DACIT construction protocol, but use twice the amount of PDMS and curing agent typically used for the devices. This will result in a PDMS master that is double the height of the usual PDMS sheet that devices are made from.
2. Line an appropriately sized (relative to the PDMS) aluminum dish such as the one suggested in the materials section of the protocol with aluminum foil.
3. Secure the PDMS master to the aluminum foil in the dish
    a. Add 5-7 drops of 10:1 uncured PDMS solution to the bottom of the PDMS master, on the side without the device indentations
    b. Flip the PDMS master and place it into the aluminum dish with the side with the device indentations facing up.
    c. Press down on the PDMS to spread out the uncured PDMS underneath the master
    d. Bake at 100°C for 10 min to cure the PDMS droplets, securing the PDMS master to the dish
4. In a disposable plastic cup, add Smooth-Cast 310 A to Smooth-Cast 310 B in a 1:1 ratio (v:v) according to the Smooth-Cast instructions.
5. Pour the resulting solution over the PDMS master and use a P200 pipette tip to remove bubbles from the device indentations
6. Put the aluminum dish with PDMS master and the Smooth-Cast solution in a desiccator to remove bubbles over 30 min.

Alternative: If a desiccator is not available, use the P200 pipette tip to dislodge bubbles from the surface of the PDMS every 5 min for 30 min.

7. After 30 min, remove the aluminum dish from the desiccator and let it sit at room temperature overnight to polymerize completely.
8. The next day, carefully remove the PDMS master from the Smooth-On mold. Use tweezers to separate the PDMS from the edge of the mold, then slowly peel the PDMS master from the surface of the mold. The Smooth-On mold (**Figure 2D**) should mimic the SU-8 master (**Figure 2C**).

Key resources table

ANTIBODIES

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Anti-PGP 9.5 (mouse monoclonal)	Bio SB	Clone: BSB-46
Anti-PGP9.5 antibody (rabbit polyclonal)	Abcam	Cat# ab15503
anti-Keratin K8/K18 (guinea pig polyclonal, serum)	PROGEN	Cat# GP11

CHEMICAL, PEPTIDES, AND RECOMBINANT PROTEINS		
REAGENT OR RESOURCE	**SOURCE**	**IDENTIFIER**
Preparation of the SU-8 master		
100 mm Silicon Wafer	Waferpro, USA	Product #: C04203
SU-8 (2005, 2100, 2150, 3050, 3005)	Kayaku Advanced Materials, Inc., USA	NA
SU-8 developer	Kayaku Advanced Materials, Inc., USA	Product #: Y020100
Disposable aluminum dishes	KichenDance.com	Cat#80063
Preparation of the Smooth-On mold		
Smooth-cast 310	www.smooth-on.com	NA
DACIT construction		
Sylgard™ 184 Silicone Elastomer, Electron Microscopy Science, Sylgard™ 184, Flash Point=121 °C (250 °F)	Electron Microscopy Sciences	Cat#24236-10
Acetone	Fisher Scientific	Cat#A18-4
Isopropanol	Fisher Scientific	Cat#S25371A
DACIT sterilization and coating		
Poly-L-lysine solution,0.1 % (w/v) in H2O	Sigma-Aldrich	Cat#P8920
Matrigel® Basement Membrane Matrix	Corning	Cat#356234
HBSS (+): HBSS with Ca^{2+}, Mg^{2+} (without phenol red)	Gibco	Cat#14025076
Dextran, Alexa Fluor™ 680; 3,000 MW, Anionic	Invitrogen	Cat#D34681
Neuron loading		
HBSS (-): HBSS without Ca^{2+}, Mg^{2+} (without phenol red)	Gibco	Cat#14170161
HEPES 1M	Gibco	Cat#15630-080
Collagenase P	Sigma-Aldrich	Cat#11249002001
Trypsin - 0.05% EDTA	Life Technologies	Cat#25-300-120
Horse serum. Heat inactivated	Atlanta	Cat#S12150
Deoxyribonuclease I [15000 units in 6 ml HBSS]	Worthington	Cat#LS002139
Cell strainer mesh 40µm	Corning	Cat#431750
Glass Pasteur pipets	Fisherbrand	Cat#13-678-20C
Neurobasal™-A Medium	Gibco	Cat#21103049
GlutaMAX	Gibco	Cat#35050061
B-27™ Supplement (50X), serum free	Gibco	17504044
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
Neural Growth factor (Ngf 2.5S Native Mouse Protein)	Invitrogen	Cat#13257019

Floxuridine (FdU)	Sigma-Aldrich	Cat#PHR2589
Molecular probes CellTracker; Red CMTPX	Invitrogen	Cat#C34552
3D spheroid embedding		
Nutragen® Type I Collagen Solution, 6 mg/ml (Bovine)	Advanced Biomatrix	Cat#5010-D
Methyl cellulose	Sigma	Cat#M6385
Lyophilized CF® Dye SE or Biotin SE (3 vials)	Biotium	Cat#92211A
Corning™ Collagen I, High Concentration, Rat Tail	Corning	Cat#354249
DPBS (10X), no calcium, no magnesium	Corning	Cat#14200166
DMEM (Dulbecco's Modified Eagle's Medium)	Corning	Cat#10-013-CV
Fetal Bovine Serum	Atlanta Biologicals	Cat#S11550
Imaging		
Paraformaldehyde, 16% w/v aq. soln., methanol free	Alpha Aesar	Cat#43368
Bovine Serum Albumin	Sigma-Aldrich	Cat#A4503

CELL LINES		
REAGENT OR RESOURCE	**SOURCE**	**IDENTIFIER**
PC12-GFP	Laboratory of Peter Lelkes	NA
4T1-mScarlet cells	Laboratory of Bojana Gligorijevic	NA

OTHERS		
REAGENT OR RESOURCE	**SOURCE**	**IDENTIFIER**
Mask Aligner	ABM USA	ABM3000HR
Spin coater	Brewer Science	CEE 200X Precision Spin Coater
Resist	Microchem	NA
Oven	Fisher Scientific	NA
Biosafety cabinet	NuAire	Labgard Class II Type A2
Biopsy punches (6 mm)	Miltix	MIL3336
Coverslips (24mm) 24x24mm No.1	Globe Scientific Inc	Model#1405-10
Sonicator	Fisher Scientific	FS-6
Plasma machine	PLASMA ETCH	PE-25 Series Plasma System
Hot plate	Fisher Scientific	Isotemp 11-100-49SH
Stereo microscope	Leica	A60
Centrifuge	Eppendorf	Centrifuge 5430R
Centrifuge	Eppendorf	Centrifuge 5804R 15amp version
10 cm dishes	Corning	Product number: 353003
3.5 cm dishes	Corning	Product number: 353001

CO_2 Incubator	ThermoScientific	Napco Series 8000 DH
Confocal Microscope	Olympus	Fluoview1000-MPE
Wide field Microscope	Nikon	Eclipse Ti2

Materials and equipment

Reagent preparation

Methyl cellulose (1.2 % low viscosity methylcellulose)

Place 2.4 g of methyl cellulose in a glass bottle with a magnetic stirring bar. Autoclave the methylcellulose and let the bottle cool to 50-60°C. Under a laminar flow hood, add 100 ml of warm DMEM medium (Gibco, Cat# 11965118) to the sterile methylcellulose and stir at 50°C until the powder is dissolved. Add 100 ml more DMEM medium and stir the mixture for three hours or until the mix reaches room temperature. Stir overnight at 4°C. The next day, centrifuge the mixture at 1878 x g for 1 h to remove precipitations. Aliquot the methylcellulose and store it at 4°C.

Matrigel aliquots

Work under the cell culture hood. Use cool tips and tubes. Thaw Matrigel stock solution on ice. Using cool P200 tips make 100 µl or 200 µl aliquots. Store at -80°C.

Cell culture media

	Stock concentration	Working concentration
Cancer cell complete medium		
DMEM, high glucose	NA	NA
Fetal Bovine Serum	NA	10%
Penicillin-Streptomycin	100X	1X
Embryonic DRG complete medium		
Neurobasal medium	NA	NA
B27	50X	1X
Glutamax	100X	1X
FdU	1000X	1X
Neural Growth factor (NGF)	25 µg/ml	25 ng/ml
Adult DRG complete medium		
Neurobasal medium	NA	NA
B27	50X	1X
Penicillin-Streptomycin	100X	1X
PC12 complete medium		

DMEM, high glucose	NA	NA
Fetal Bovine Serum	NA	7.5%
Horse Serum	NA	7.5%
Neural Growth factor (NGF)	100 µg/ml	50 ng/ml

Timeline

Steps / Days	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7		Day 8		Day 9		Day 10	
	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M	A
DACIT construction																				
Clean SU-8 master																				
Make PDMS solution																				
Cure PDMS																				
Cut out and punch PDMS Devices																				
Clean freshly cut PDMS Devices																				
Clean 24 mm coverslips																				
Permanently bond PDMS device to coverslip																				
DACIT sterilization and coating																				
DACIT sterilization																				
DACIT coating PLL																				
DACIT coating Matrigel																				
Neuron loading																				
Primary Culture: Dissection and Dissociation																				
Adding neural cells to DACIT																				
Primary Culture: Change medium																				
Cancer cell loading 3D																				
Hanging Drop																				
Embed spheroids in DACIT																				
Fix																				
Immunofluoresence																				
Perm/Blocking/Primary antibody																				
Secondary antibody																				
Imaging																				

Step-by-step methods details
DACIT construction

Timing: 2 days

The result of this protocol will be a fully constructed DACIT. This protocol assumes one batch of PDMS devices is being made from one SU-8 master (**Figure 2C**). If using multiple masters or Smooth-On molds, change quantities accordingly. In this protocol, the PDMS portion of the DACIT before it has been attached to the coverslip will be referred to as a PDMS device. The PDMS device adhered to the coverslip will be referred to as a DACIT.

Day 1

1. Clean SU-8 master
    a. Rinse the SU-8 master with acetone, isopropanol, and then DI water to remove any dust particles or PDMS residue from previous use.

    Note: If casting PDMS onto a Smooth-On mold, clean with isopropanol and DI water only, not acetone.

    b. Dry the SU-8 master completely with compressed air.
    c. Line the aluminum dish with aluminum foil and place the clean master in the dish. Cover it until ready to add PDMS.

    Alternative: To permanently bond the master to the aluminum dish, add 5-7 drops of 10:1 PDMS solution to the bottom of the SU-8 master. Press the bottom of the master into the aluminum dish to spread out the PDMS solution and bake for 10 min at 100°C to cure the PDMS.

2. Prepare, pour and cure PDMS (**Figure 3A.i)**
    a. In a plastic cup, weigh out the Sylgard 184 elastomer base and with a transfer pipette add the Sylgard 184 elastomer curing agent in a 10:1 ratio

    Note: Smooth-On mold: 30 g elastomer base + 3 g of curing agent; Chrome mask: 45g elastomer base + 4.5 g of curing agent, Adjust accordingly)

    b. Mix the base and curing agent together using the transfer pipette until the solution is homogeneous.
    c. With one hand, use a P200 pipette tip to apply pressure to the center of the SU-8 master in the dish. With your other hand, pour the PDMS onto the SU-8 master around the pipette tip. Apply pressure with the pipette tip as necessary to minimize the amount of PDMS that goes under the master.

    Note: If the master is permanently bonded to the dish, using the P200 pipette tip to apply pressure to the master is not necessary.

    d. Remove the bubbles by placing the aluminum dish with the master and 10:1 PDMS into a desiccator for 10 min. After, use compressed air on the PDMS to remove the remaining bubbles on the surface.

    Alternative: If a desiccator large enough for the master and an aluminum dish is unavailable, remove the bubbles while the PDMS is in the plastic cup by placing it in a desiccator for 5 min. Repeat this step until few or no more bubbles remain (~3-5 times).

e. Cure the PDMS by covering the dish with aluminum foil, and leave in the oven at 65 °C overnight.

Note: If there are any bubbles present in the PDMS, pop them with a pipette tip, or guide them to the edge of the dish so that they are not over the channels or microgrooves of the device.

Day 2

3. Cut out and punch PDMS devices (**Figure 3A, ii**)

Note: Cut out the devices in a laminar flow hood to minimize particles that could become stuck to the surface of the PDMS device

a. Let it cool to room temperature (45-60 min)
b. Remove the cured PDMS from the SU-8 master, and wrap it with aluminum foil.
c. In the laminar flow hood, unwrap the PDMS and place it on a clean cutting mat.
d. Using 6 mm biopsy punches, punch holes where the circles are at the four corners of the devices that will act as the wells.

Critical: Be consistent with how you line up your tool for hole-punching so the holes are as evenly spaced and reproducible as possible

Critical: Use sharp biopsy punches to minimize creating PDMS debris

e. Cut the devices into 23 mm squares using a ruler to aid in creating straight lines.

Critical: Leave at least 2 mm between the wall of the well and the edge of the device to avoid leaking

Note: Keeping the devices wrapped in aluminum foil prevents them from getting dusty.

f. Check the quality of the devices under a microscope

4. Clean coverslips and freshly cut PDMS devices (**Figure 3A, iii**)

a. Clean 24 mm coverslips.
   i. Leave the coverslips in a bath of 1M HCl for at least 1 hr.
   ii. Rinse the coverslips in distilled water.
   iii. Swirl the coverslips by hand in a bath of isopropanol for 1 min.
   iv. Ultrasonicate for 10 min in an isopropanol bath.
   v. Place in oven at 65°C for 5-10 min or until the coverslips are dry.
   vi. Keep them in a closed container, such as a 10 cm tissue culture dish, lined with aluminum foil.

b. Clean freshly cut PDMS devices
   i. Submerge PDMS devices in isopropanol and swirl by hand for 1 min.
   ii. Transfer the devices to a bath of 70% ethanol and swirl by hand for 1 min.
   iii. Ultrasonicate devices for 10 min in a 70% ethanol bath.
   iv. Use compressed air to remove liquid from the devices. Be gentle around the microgrooves
   v. Place the devices in the oven at 65°C for 10 min or until the devices are dry. Flip the devices to completely dry both sides.

vi. Once the devices are dry, keep them in a closed container, such as a 10 cm tissue culture dish, lined with aluminum foil.

Good Pause Point

5. Permanently bond the PDMS device to a coverslip (**Figure 3A,.iv**)
   a. Place devices and coverslips in the plasma machine.

   Critical: Devices must be channel/microgrooves side up in the plasma machine. Only the side facing up will be exposed to plasma and gain the surface changes necessary for bonding.

   b. Plasma coat coverslips and devices at 120-180 mTorr for 2 min.

   Note: Timing may be dependent on the plasma machine.

   c. Remove PDMS devices and coverslips from the plasma machine and gently place the coverslip on the PDMS device
      i. Attach the plasma-coated side of the coverslip (the side that was facing up in the plasma machine) to the plasma-coated side of the PDMS device.

      Critical: Crushing the microgrooves is very easy to do, and will probably happen relatively often when starting out, the key to avoiding crushing them is to be as gentle as possible when putting the devices together.

      Note: You should be able to see the attachment happening between the coverslip and the PDMS as the disappearance of air between the two.

      ii. Wait 3 min after coverslip attachment before moving onto the next step.

   d. Check the DACITs under a microscope to ensure that the microgrooves have not been crushed in the process of putting it together and to check the seal around the wells and channels of the device.
      i. Gently apply pressure to the microgrooves using the tips of the tweezers to help seal them to prevent DACIT leakage later in the protocol.
      ii. Check the seal around the wells of the device by checking for air pockets around the wells. Air pockets will look like an area that is a slightly different color than the surrounding area. If there are air pockets, use tweezers to apply concentrated pressure over the air bubble while looking through the microscope to ensure the air pocket disappears.

      Note: Air bubbles can get caught around the main channels as well, so check them under the microscope, and use the same method as described in step (i) to remove them.

   e. To strengthen the plasma bond, put the DACIT on a hot plate set to 100°C for 1 min then return them to their closed container to prevent anything from settling on them.

   Critical: Do not leave the DACIT on the hot plate for longer than 1 min or the glass may crack.

DACIT loading strategies

Timing: 10 min – 1 h depending on the protocol

This protocol describes how to add liquids to the DACIT to achieve different outcomes and is for referencing throughout the rest of the protocol paper. When adding or removing a solution to/from the DACIT put the tip of a P200 pipette at the bottom of the wall of the well opposite the channel. Do not remove directly from the channel as this can crush the microgrooves. See **Figure 2A** for the label of the wells.

Compartmentalization.

For testing medium compartmentalization, medium with 3 kDa dextran was added to the axon side of DACIT and incubated for 48 hr at 37°C. Following the Compartmentalization protocol, DACIT design grants medium compartmentalization for 48 hr (**Figure 4A**, left panel) which is approximately the maximum time that can be left without refreshing.

1. Loading solution into the DACIT
    a. Add 200 µl of the solution to well A
    b. Wait 1 min
    c. Add 150 µl of the solution to well C
2. Removing the solution from the DACIT
    a. Slowly remove all solution from well D
    b. Slowly remove all solution from well B

Gradient formation

To test gradient formation, medium with 3 kDa dextran was added to the axon side of DACIT. Using the Gradient formation protocol, a gradient was established in the first 6 hr and it was maintained for 48 hr (**Figure 4A**, right panel).

1. Loading solution into the DACIT
    a. Add 200 µl to well C
    b. Wait 1 min
    c. Add 150 µl to well A
2. Removing solution from DACIT
    a. Slowly remove all solution from well B
    b. Slowly remove all medium from well D

Washing DACIT

1. Loading solution into DACIT
    a. Add 200 µl of PBS into well A
    b. Wait 5 min (first wash/coat only)
    c. Add 200 µl to well C
2. Removing PBS
    a. Slowly remove all PBS from well D
    b. Slowly remove all PBS from well B

DACIT sterilization and coating

Timing: 24 h

This protocol describes how to sterilize and coat the DACIT to prepare it for cell culture. This protocol and all of the following protocols must be performed in the biosafety cabinet except for step 1a

1. Sterilize DACITs
    a. Add 70% ethanol to DACITs following the Compartmentalization protocol from the DACIT loading strategies section.
    b. If the devices are not leaking, transfer them to the biosafety cabinet and leave them under UV light for 15 min.
    c. Wash 3 times with PBS as following the Washing DACIT protocol from the DACIT loading strategies section.
2. Coat DACITs with Poly-L-lysine (PLL) solution
    a. Add 50 µg/ml PLL solution to DACIT following the Compartmentalization protocol using half of the volume.
    b. Let the PLL incubate in the DACITs overnight at room temperature
    c. Remove PLL from the DACITs following the solution removal portion of the Compartmentalization protocol
    d. Wash the DACITs with PBS two times following the Washing protocol

Good Pause Point: At this point, the device may be stored in an incubator at 37°C for up to a week with DPBS P/S solution in the wells

3. <u>Matrigel Coating</u>

Note: We recommend using the DACITs within 0-2 days of coating. If you need to use them later, store at 4°C with PBS until ready for use.

Note: During all the protocols use cool tips. Put a box of sterile P200 tips at -20°C the previous day.

Note: Keep the medium and the Matrigel on ice at all times and handle the Matrigel with cool pipette tips.

    a. Thaw Matrigel aliquots at 4°C
    b. Dilute Matrigel in a 1:10 or 1:20 ratio on PBS (-) with chilled medium and keep on ice.
    c. Remove PBS from DACITs following the removal portion of the Washing protocol
    d. Add chilled medium to the DACITs following the loading section of the Washing protocol with half the volume (100 µl), and skip the waiting time.
    e. Remove the cold medium following the removal portion of the Washing protocol
    f. Add the diluted Matrigel solution to the DACITs following the loading portion of the compartmentalization protocol but using half the volumes and waiting 5 min instead of 1 min.

    Note: Keep the DACITs on ice for the 5 min wait so the Matrigel does not start to polymerize.

    g. Let the Matrigel solution incubate in the device for 30 min in an incubator at 37°C or for an hour at room temperature
    h. Remove the solution following the removal portion of the Compartmentalization protocol.
    i. Wash three times following the Washing protocol.

j. Add medium following the Compartmentalization protocol and put the DACIT in an incubator at 37°C until cell loading

Good Pause Point

Neuron loading

Timing: 5 hr (including dissection)

This protocol describes how to add cells to the neuron side of DACIT. As a neuron model, we used the pheochromocytoma cell line PC12 or primary DRG neurons recovered from adult mice or embryonic rats. Axon frequency and length vary according to the number and type of neurons. We observed that PC12 cells project short axons into the microgrooves that rarely reach the axon channel (**Figure 4B**, top panel and **Figure 4C**). Besides, most of the axons from a primary culture of dorsal root ganglia (DRG) neurons reach the axon channel (**Figure 4B**, medium and bottom panels).

Note: Pipet the DRG cell solution and centrifuge minimally, the disturbance can negatively affect the health of the cells.

Mouse DRG dissection and dissociation

Timing: 4.5 h

DRG dissection and dissociation from adult mice were performed as described in previously[14]. Briefly, mice were sacrificed, the spinal cord was removed, and around 30 DRGs were recovered per mouse. Recovered DRGs were digested with Collagenase P (5 mg/ml) per 45 min at 37°C, followed by trypsin/EDTA for 5 min at 37°C. Trypsin was inactivated with horse serum in a complete Neurobasal medium and the cells were disaggregated using a polished pipet. Cells suspension was filtered through a 40µm cell strainer mesh to remove cell clusters and through a bovine serum albumin (BSA) 10% solution to remove non-neurons cells. Finally, cells were concentrated by centrifugation and resuspended at a 125 000 cells/ml density in adult DRG complete medium to plate in the DACITs.

Embryonic rat DRG dissection and neuron dissociation

Timing: [2 h]

E16 rat embryos were removed and DRGs were collected and placed on cold HBSS. Embryonic rat DRGs were digested first with Collagenase (10kU/ml) for 15 min at 37°C which was neutralized with fetal bovine serum (FBS), and after with trypsin for 15 min at 37°C. Trypsin was inactivated with FBS and washed out by centrifugation. Dissociated DRGs were triturated on Neurobasal medium using pipet tips (P1000 and then P200) before quantification. Cells were resuspended on complete Neurobasal medium to load in the DACITs.

Neuron loading

Timing: [30 min]

1. Prepare a cell suspension of 1.25 x10^6 PC12 cells/ml or 250,000 DRG cells /ml in the corresponding cell culture medium.
2. Remove media from coated DACIT following the removal portion of the Compartmentalization protocol.
3. Using the P20 pipette tip, add 8 - 10 µl of cell suspension directly into the neuron channel from well A (See **Figure 2A**).

Note: This is easiest to do if you are holding the DACIT by the edges with your fingers.

4. Tilt the DACIT against the 10 cm petri dish so that it is sideways, to promote cell adhesion closer to the microgrooves. Carefully transfer the closed 10 cm dish with the DACITs to the incubator for 7 min.

Critical: Do not keep the DACITs this way for longer than 10 min or the cells may squeeze into or under the microchannels.

5. Put the DACIT back into a flat position and put it into an incubator for 15 min to allow the cells to attach.
6. If required, prepare the medium for NGF gradient formation while the cells are attaching. Media with NGF should be prepared fresh because NGF is unstable at 37°C.
7. Add medium to the DACIT using the protocol for compartmentalization or gradient formation depending on your experimental needs.

Note: An NGF gradient can be used to expedite axon growth through the microgrooves.

Cancer cell loading – 2D

Timing: 1 hr

Once axons have grown through most of the microgrooves and entered the axonal compartment, cancer cells can be embedded in the device for cancer cells-axon experiments (see **Figure 4B**, left panel).

1. Prepare cancer cell suspension with 2,500 cancer cells in 10 µl (250,000 cells/ml) in a complete cancer cell medium.
2. Remove the medium from well D of the axon side of the device.
3. Wash the axon side of the DACITs 2 times with HBSS calcium and magnesium-free following the Washing protocol to remove NGF.
4. Add 8 – 10 µl of the cell suspension to the axon channel.
5. Put the DACIT in the incubator for 15 min to allow cells to attach to the channel.
6. Add more complete neurobasal medium to the neuron side if necessary, so that there is 200µl in the neuron side.
7. Add 140 µl of complete cancer cell medium to cancer cell side.

Critical: This 50 µl volume difference is what will allow for fluidic isolation of the cancer cell medium.

8. Incubate the devices with neurons and cancer cells at 37°C and 5% CO_2 for as long as the experiment being done requires.

Spheroid embedding in 3D

Timing: 3 hr

Using the SU-8 preparation protocol presented here, it is possible to reach a channel between 400-500 µm height (**Figure 2D**) which allows us to analyze the invasion of a cancer cell spheroid of around 200 µm diameter into the extracellular matrix. A proper invasion protocol requires that spheroids are completely embedded in the matrix (**Figure 5B**) and close enough to the microgrooves to be reached by axons (**Figure 5C-D).** See Figure 4A and the timeline to properly coordinate neuron axon growth and spheroid formation.

Cancer cell spheroid preparation

Spheroids were prepared as described previously[15]. Briefly, 4.8 mg/ml methylcellulose, 1000 cancer cells/ml diluted in DMEM complete cell culture medium, and 20 µg/ml nutragen were mixed in an Eppendorf tube on ice. Then, 40 µl drops of the mixture were placed on the lid of a

cell culture dish, to form spheroids containing 50 cancer cells each. Spheroids were kept in the incubator for 24 – 36 h with PBS at the bottom of the plate to prevent them from drying out. Spheroids with best morphology were selected under the microscope and washed three times with warm DMEM complete medium before embedding.

Spheroid embedding

The following protocol is being described assuming 50 µl of embedding solution is being prepared. Update the volumes depending on the number of DACITs.

1. Wash the spheroids
    a. Collect spheroids deemed using a P1000 pipette tip and put them in a tube with warm cancer cell-medium
    b. Wait 5 min for the spheroids to fall to the bottom of the falcon tube
    c. Remove the medium by pipetting from the top without disturbing the spheroids at the bottom. Don't aspirate.
    d. Add fresh cancer cell medium.
    e. Repeat b-d washes four times.

2. Prepare the embedding solution using amounts from the table.

Note: Reagents must be kept on ice for the entirety of the protocol

Note: Add reagents from the embedding solution table in the order and amounts that they appear in the table. Wait to add NaOH until right before adding the spheroids

	Stock concentration	**Work solution**	**Volume**
Collagen	9.5 mg/ml**	2 mg/ml	10.52 µl
Matrigel	NA	NA	10.52 µl
PBS	10 X	1 X	5 µl
Complete cancer cell medium	NA	NA	Gauge to 50 µl
NaOH* **	1 N	NA	0.247 µl
Medium with spheroids	NA	NA	10 µl
Final volume*			**50 µl**

* Use a minimum solution volume of 50 µl to avoid pipetting error

** The concentration of the collagen varies. Adapt the volume required according to the stock concentration.

*** The volume of NaOH depends on the volume of collagen. Multiply the collagen volume by 0.023 to obtain the volume of 1N NaOH

Optional: Collagen can be labeled using succinimidyl ester (SE)-405 by incubating 1 mg of collagen with 0.13 µg SE-405 for 20 min on ice.

3. Prepare DACITs for embedding
    a. Remove the medium from the axon side of the device and discard it.
    b. Remove the medium from the neuron soma side of the device and keep it in the incubator.
4. Add spheroids to DACIT

    a. Mix the embedding solution with spheroids thoroughly before starting the embedding process
    b. Use a P20 pipette tip to measure 6 µl of embedding mixture with spheroids which should be slightly visible. Place the P20 pipette tip at the entrance of the axon channel and slowly add the embedding solution to the channel
5. Flip DACIT while collagen and Matrigel polymerize
    a. Place the device upside down on aluminum foil on a 10 cm culture dish.
    b. Place the dish in the incubator and let it sit for 1 min
    c. Take the devices out of the incubator and in the hood slowly flip them onto their opposite side
    d. Repeat steps 6a-b five times.
    e. Let collagen polymerize for another 15 min
6. Add medium to DACIT
    a. Supplement the medium kept from the neuron side of the devices with fresh medium so that there is enough medium to add 200µl to each neuron soma channel.
    b. After adding 200µl to the neuron soma side, add 75µl of complete cancer cell medium to each well on the axon side of the device.

Imaging

Timing: 1 day

Immunofluorescence

To perform immunofluorescence of neurons and spheroids in DACIT, we followed the previously published protocol[16]. Briefly, the medium was removed from DACIT and the cells were washed once with PBS. Cells were incubated with a fixing/permeabilizing solution (4% PFA with 0.5% Triton X-100 in PBS) for 10 min at room temperature. The solution was removed, and cells were incubated with fixing solution (4% PFA in PBS) for 20 min at room temperature. After fixation, cells were washed three times with washing solution (PBS containing 0.05% Tween-20), and unspecific sites were blocked with blocking solution (1% FBS, 1% BSA in PBS) for 1 hour at room temperature. The blocking solution was removed and cells were incubated overnight at 4°C with the primary antibodies diluted in the blocking solution. The next day, the primary antibody was removed, and the cells were washed three times with washing solution for 10 min each at room temperature.
Then, the cells were incubated for 1 h at room temperature with the secondary antibodies diluted in blocking solution. After incubation, cells were washed three times with the washing solution for 10 min each at room temperature. DACITs were stored in PBS in a humidity chamber at 4°C.

Imaging

To mount and image DACITs, plastic single DACIT and 6-device holders were designed and 3D printed (**Figure 2F**). Files containing the designs are available in the Resource availability section.

Expected outcomes

In the “before to begin” section, we describe the protocol to obtain a SU-8 master with channels of around 500 µm height to fit cancer cells spheroids (**Figure 2E**). We also included a Smooth-On mold protocol to duplicate the SU-8 master in a cost-effective way.

In the section 'step-by-step methods details', we describe in detail how to prepare DACIT. This protocol must be mastered to ensure a high success rate. Critical points are highlighted, and common problems are addressed in the troubleshooting section. Next, we described how to load neurons in DACIT. It is expected that loaded neurons project their axons to the axon side of DACIT. The frequency and the length of the axons depend on the health of the cells and on the type of neurons you are loading. Finally, we described how to load cancer cells in 2D or in 3D as cancer cell spheroids. Successful loading of cancer cell spheroids in the presence of axons requires practice. Using the strategy detailed here, it is expected to monitor cancer cell invasion in the presence of axons.

Quantification and statistical analysis

1. **Compartmentalization analysis**
   Confocal time-lapse images were analyzed using Fiji ImageJ2 (version 2.9.0/1.53t). Briefly, time-lapse Z-stack was projected using the maximum intensity projection and the intensity decay by photobleaching was corrected by bleach correction using the simple ratio method and a 0.0 background intensity. Identical regions of interest were selected in the neuron soma and the axon/cancer cell channels and the mean intensity signal of Dextran-Alexa-680 was determined during the time-lapse.

2. **Axon quantification**
   The length of the axons was determined with Fiji, ImageJ2 (version 2.9.0/1.53t). Briefly, z-stack images obtained in the confocal microscopy were projected in a 2D plane using the Maximum intensity projection. Then, axons were tracked using the NeuronJ plugin[17]. The length of the axon was determined from the beginning of the microgrooves to the axon terminal, or the longest length observed in the picture.

Troubleshooting

Problem 1:
The cured device has bubbles
Step:
DACIT construction, Step 2: Prepare, pour, and cure PDMS
Potential solutions

- Increase the time on the desiccator.
- After pouring the uncured PDMS into the SU-8 master, move the bubbles with a tip to the border of the edge of the master.

Problem 2:
PDMS devices have varying heights.
Step:
DACIT construction, Step 2: Prepare, pour, and cure PDMS
Potential solution
Uncured PDMS has leaked under the master. To avoid this, apply pressure to the master while pouring the PDMS into the dish using a P200 tip. Alternatively, permanently bond the master to the bottom of an aluminum dish with PDMS.

Problem 3:
The cured PDMS sticks to the SU-8 master
Step:
DACIT construction, Step 2: Prepare, pour, and cure PDMS
Potential solution

- Let the cured PDMS cool for 1 h or until cool to the touch before demolding.
- Verify the temperature of the oven. PDMS firmly attaches to the master if cured at > 60°C. Cure the PDMS at 60°C or below. To peel off stuck PDMS, let the master sit on acetone over the weekend.

Problem 4:
The added media leaks between the coverslip and the PDMS
Step:
DACIT construction, Step 3: Cut out and punch of devices
Potential solution

- This occurs because the punches are too close to the edge of the device. To solve this be sure you leave between 1-2 mm between the well and the edge of the device. If it is necessary, use bigger coverslips to have the channels further from the edge or punch the holes for wells closer in towards the microgrooves
- After bonding, use tweezers to apply concentrated pressure at the borders of the device to remove air pockets.

Problem 5:
There is dust and other debris on the PDMS portion of the device and coverslip even after cleaning
Step:
DACIT construction, Step 4: Clean coverslips and freshly cut PDMS devices
Potential solution

- Cut out devices in the biosafety cabinet and handle them outside of the BSC minimally
- Don’t use dull products to cut the PDMS (box openers/ biopsy punches should be sharp)
- Use scotch tape to clean the devices right before plasma bonding

Problem 6:
The microgrooves are crushed
Step:
DACIT construction, Step 5: Permanently bond the PDMS device to a coverslip
Potential solution
Do not apply weight to the PDMS device after plasma bonding.

Problem 7:
PDMS is detaching to the coverslip after plasma treatment.
Step:
DACIT construction, Step 7: Permanently bond PDMS device to coverslip
Potential solution
- The conditions of the plasma machine can vary depending on the machine. Standardize the proper conditions.
- Verify that the correct side of the coverslip and PDMS device are facing up during the plasma treatment.
- Plasma treatment activates the PDMS and coverslip surfaces temporally. Perform the bonding within 5 min after plasma treatment.

Problem 8:
Some axons growth under the microgrooves.
Step:
DACIT construction, Step 7: Permanently bond PDMS device to coverslip
Potential solution
- Never skip the step where you put the newly made DACITs on a hot plate at 100°C for 1 min to strengthen the bond between PDMS and coverslip.
- Gently press on microchannels with tweezers while viewing freshly bonded DACIT with a light microscope to ensure microgrooves are attached.
- Be sure to properly clean the PDMS devices and the coverslips before bonding. Dust under the microgrooves affects proper sealing.

Problem 9:
There are few or no axons in the microgrooves.
Step:
Protocol for neuron-side cell loading: Neuron loading.
Potential solution
This can occur because there are not enough neurons close enough to the microgrooves
- Add more neurons to the neuron side of the DACIT.
- After loading neurons and before adding a complete neurobasal medium, keep the device sideways for 5 – 7 min to encourage cells to go close to the microgrooves. Be careful not to wait more than 10 min to avoid cells blocking the microgrooves
- Generate an NGF gradient from the axon side following the Gradient formation protocol to promote axonogenesis.

Problem 10:
Spheroids spread across the bottom of the dish while invading (**Figure 5E**).
Step:
Protocol for cancer cell-side cell loading – 3D spheroid embedding

Potential solution
Flipping the DACIT is key to avoid the spheroid touching the bottom. Standardize the intervals of the flipping according to the size of the spheroid and the density of the collagen.

Resource availability

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bojana Gligorijevic, bojana@temple.edu.

Materials availability
This study did not generate any new unique results.

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Author contributions

Conceptualization: I.V.Q., V.A., E.T., B.G.; Data acquisition, analysis, and interpretation: I.V.Q., K.A., V.A., N.H., B.G.; Writing: I.V.Q., K.A., V.A., E.T., G.T. and B.G.; Supervision: E.T., G.J. and B.G.

Declaration of interests

The authors declare no competing interests.

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Figure legends

Figure 1. Design of the DACIT layers. (**A**) The overlay of microgrooves (blue) and the channel (red) layers; (**Ai**) a closeup of the microgrooves connecting the left and right channel. (**B**) Microgroove layer design, (**C**) Channel and wells layer design.

Figure 2. DACIT fabrication. (A) A schematic showing DACIT 3D view (left panel), top (middle), and side view (right panel). Soma compartment (yellow) and axonal compartment (magenta) each contain two wells where solutions can be added (*A, C*) or removed (*B, D*). (**B**) Main steps in preparation of the SU-8 master: spinning, mask alignment, baking, and UV exposure. (**C**) Final look of SU-8 master and (**D**) Smooth-On mold. Scale bar = 2 cm. **(E**) A brightfield image of the DACIT cross-section shows the channel height measures 500 microns. The dotted white line represents the position of the coverslip. Scale bar 200 µm. (**F**) 3D-printed holders designed to mount and image a single DACIT device (orange) and six devices at a time (white). Scale bar = 2 cm.

Figure 3. Scheme of the DACIT construction steps. (**A**) Steps to build DACIT from the SU-8 or the Smooth-on mold master and (**B**) Frequent problems that can occur in each step. Potential solutions are described in the troubleshooting section.

Figure 4. DACIT grants medium and cellular compartmentalization. Medium compartmentalized (**A**, left panels) or in gradient (**A**, right panels) was tested by adding 3 kDa dextran on the axonal compartment as detailed in the DACIT loading strategies section. The distribution of 3kDa dextran was monitored every hour for 48 hours while incubated at 37°C 5% CO2. Pseudo-color images (**A**, top panels) are snapshots showing the distribution of the dye after 24 h of incubation. The scale bars equal 200 µm. (**B**) Shows inverted images of GFP (for PC12-GFP cells) or cell-tracker labeled cells (Primary culture of DRG cells). Cells were loaded in the neuronal compartment and axons grew in the presence of an NGF gradient. A close view of axons growing into the microgrooves (**B**, left panel. Scale bar: 50 µm) and a larger view showing axons growing into the axon channel (**B**, right panel. Scale bar: 200µm) are presented. (**C**) Quantification of the length of axons in the microgrooves.

Figure 5. 3D Invasion Assay in DACIT. (**A**) A schematic showing the experimental timeline of 3D spheroid invasion in DACIT. (**B**) 3D Imaris reconstruction of two 4T1-mScarlet spheroids (magenta) embedded in 405-collagen:Matrigel mix (blue). Scale bar 200 µm. (**C**) A brightfield image of an

invading spheroid at 24h. Scale bar 200 µm. (**D**) Fixed 4T1-mScarlet spheroid (magenta) with PGP9.5+-immunolabeled axons (white). Scale bar: 100 µm (**E**) Brightfield images of a spheroid properly embedded in 3D (top) and a spheroid at the bottom of the channel (bottom). Scale bar: 200 µm.

Figures

Figure 1.

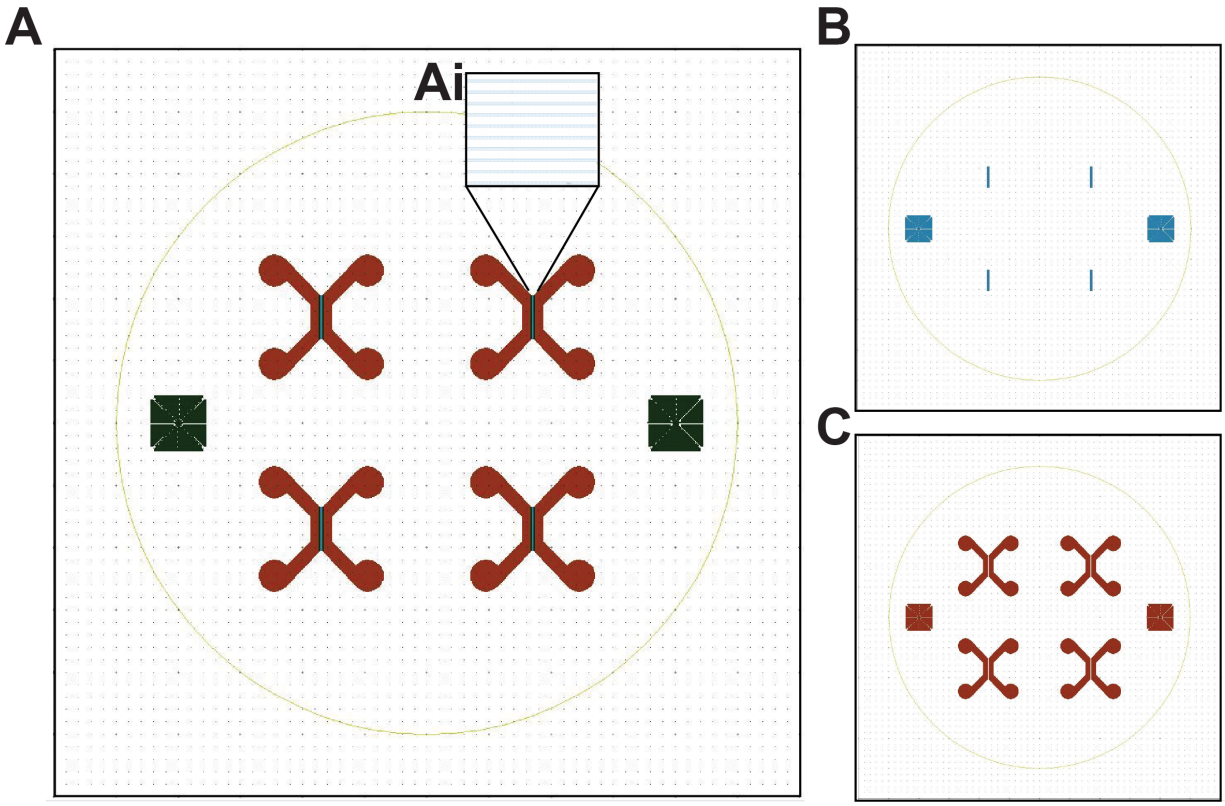


Figure 2.

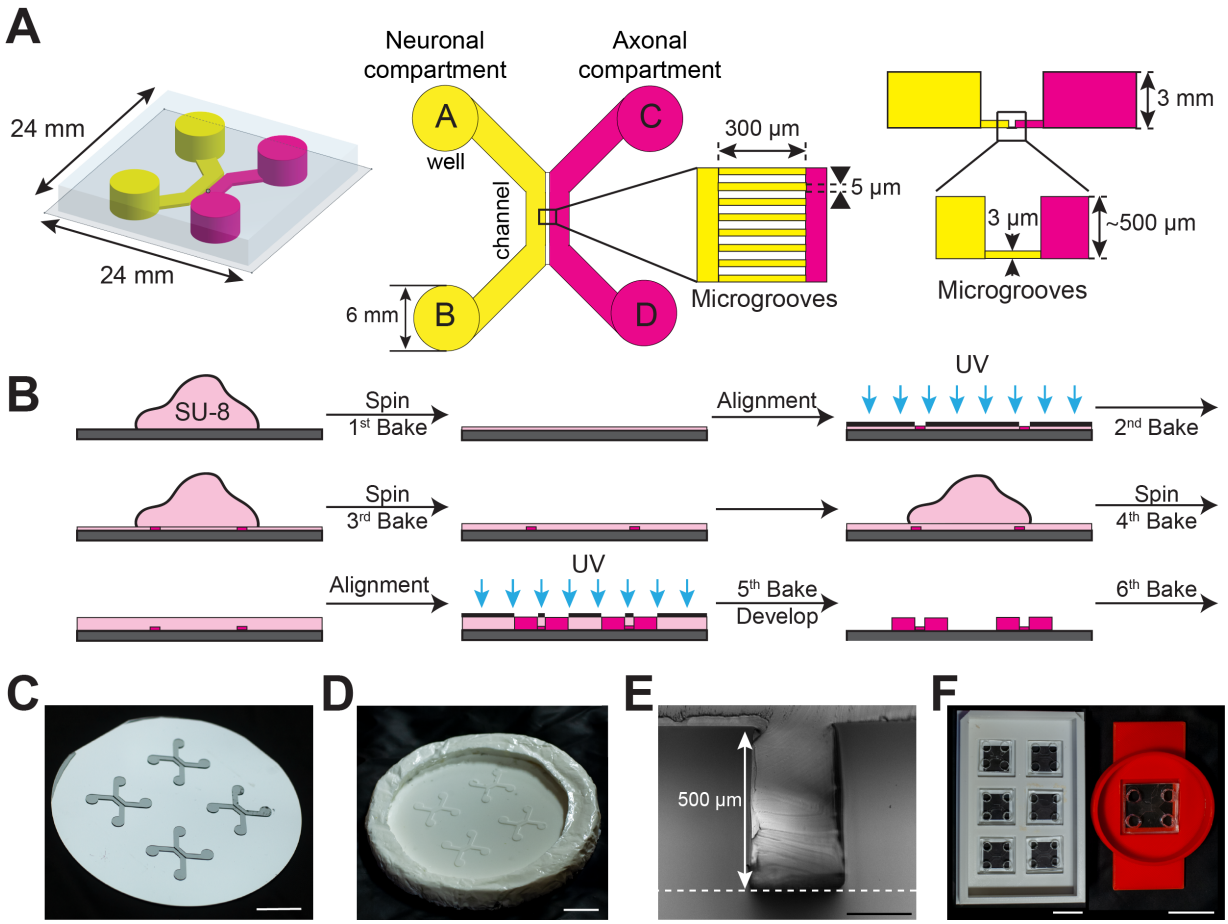
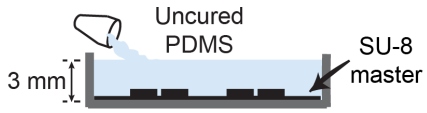
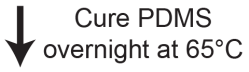

A
24 mm
24 mm
Neuronal compartment
Axonal compartment
A
well
B
C
D
channel
6 mm
300 µm
5 µm
Microgrooves
3 mm
3 µm
~500 µm
Microgrooves
B
SU-8
Spin
1st Bake
Alignment
UV
2nd Bake
Spin
3rd Bake
Spin
4th Bake
Alignment
UV
5th Bake
Develop
6th Bake
C
D
E
500 µm
F

Figure 3.

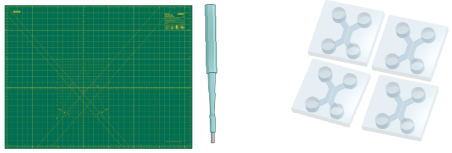
A

i. Prepare and pour PDMS

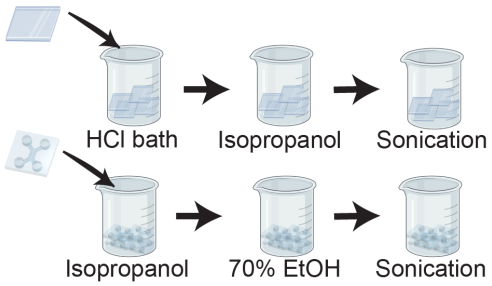




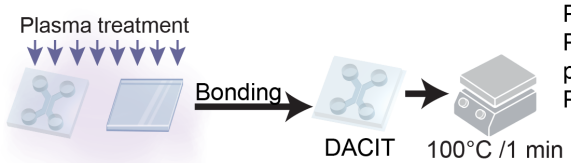
ii. Cut out and punch devices



iii. Clean coverslips and freshly cut PDMS devices



iv. Permanentely bond



B

Problem 1. The cured device has bubbles
Problem 2. PDMS devices have varying heights

Problem 3. The cured PDMS sticks to the SU-8 master

Problem 4. The added media leaks between the coverslip and the PDMS

Problem 5. There is dust and other debris on the PDMS portion of the device and coverslip even after cleaning

Problem 6. Crushed microgrooves
Problem 7. PDMS is detaching to the coverslip after plasma treatment
Problem 8. Some axons grow under the microgrooves.

Figure 4.

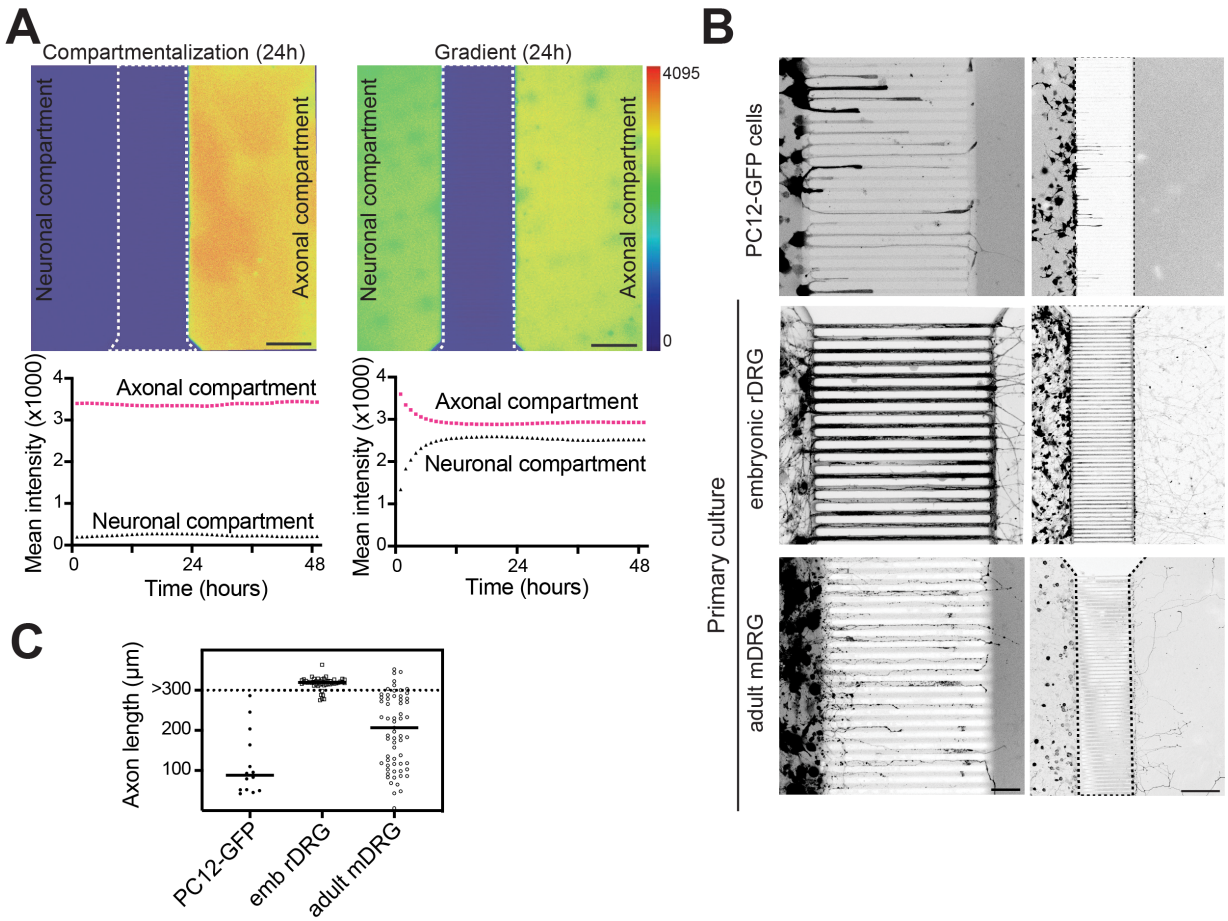
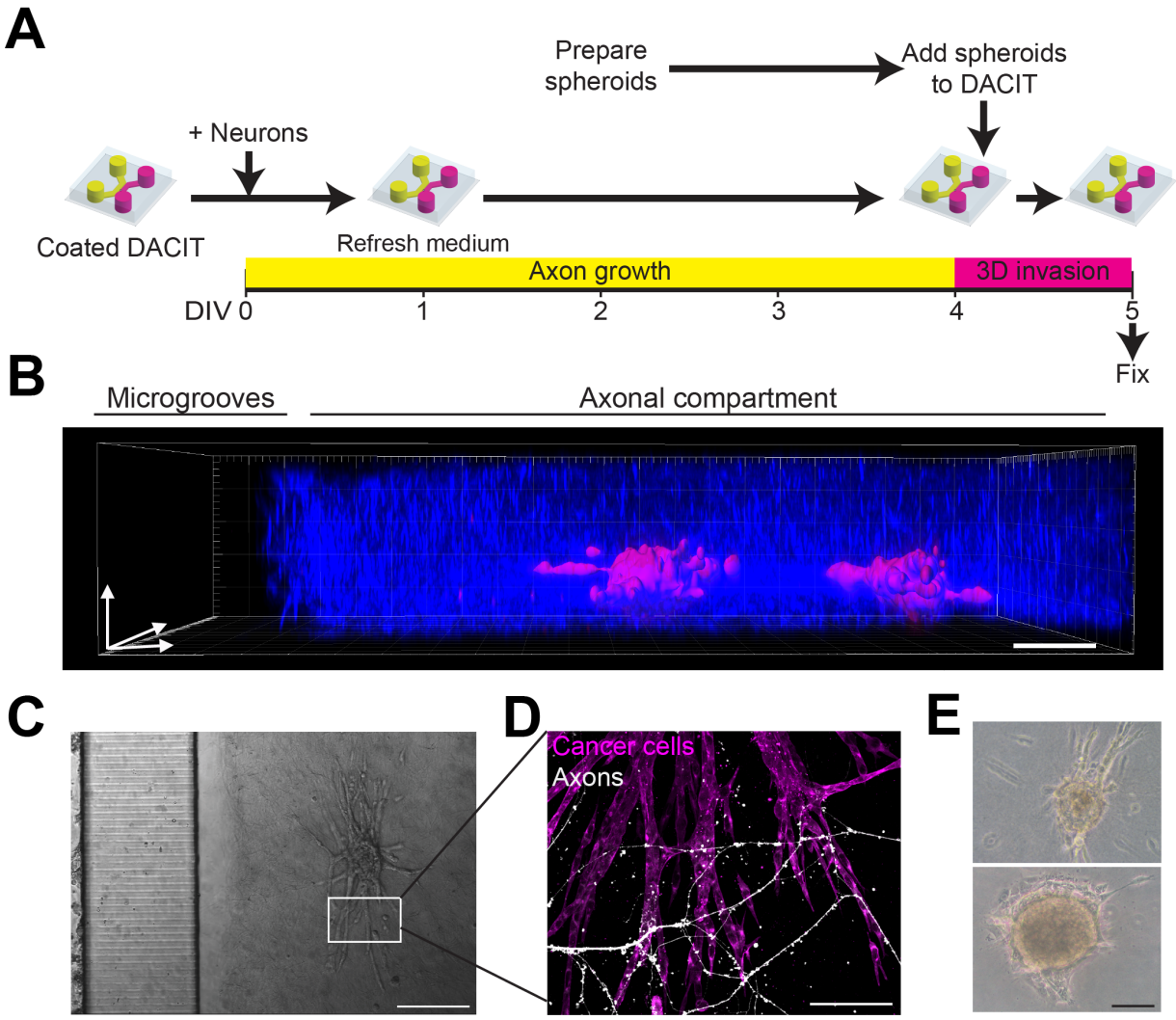

A
Compartmentalization (24h)
Gradient (24h)
Neuronal compartment
Axonal compartment
4095
0
Mean intensity (x1000)
Axonal compartment
Neuronal compartment
Time (hours)
B
PC12-GFP cells
Primary culture
embryonic rDRG
adult mDRG
C
Axon length (µm)
>300
200
100
PC12-GFP
emb rDRG
adult mDRG

Figure 5.


A
Prepare spheroids
Add spheroids to DACIT
+ Neurons
Coated DACIT
Refresh medium
Axon growth
3D invasion
DIV 0
1
2
3
4
5
Fix
B
Microgrooves
Axonal compartment
C
D
Cancer cells
Axons
E