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A microfluidic device to investigate axon targeting by limited numbers of purified cortical projection neuron subtypes

Suzanne Tharin^{1,4}, Chandrasekhar R. Kothapalli^{1,5}, Pembe Hande Ozdinler^{2,3,6,7}, Lincoln Pasquina^{2,3,6}, Seok Chung^{5,6,8}, Johanna Varner^{5,9}, Sarra DeValence^{5,9}, Roger Kamm^{5,10-12}, Jeffrey D. Macklis^{2,3,11,12}

¹Joint first authors contributed equally to this work.

²Department of Stem Cell and Regenerative Biology and Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA.

³MGH-HMS Center for Nervous System Repair, Departments of Neurosurgery and Neurology, Program in Neuroscience, Harvard Medical School; Nayef Al-Rodhan Laboratories, Massachusetts General Hospital, Boston, MA 02114, USA.

⁴Current address: Department of Neurosurgery, Cleveland Clinic Foundation, 9500 Euclid Avenue S40, Cleveland, OH 44195

⁵Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

⁶Joint second authors contributed equally to this work.

⁷Current address: Department of Neurology, Northwestern University, Chicago, IL, USA.

⁸Current address: Department of Mechanical Engineering, Korea University, Korea.

⁹Authors contributed equally to this work.

¹⁰Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

¹¹Joint senior authors contributed equally to this work.

¹²Corresponding authors: rdkamm@mit.edu, jeffrey_macklis@hms.harvard.edu.

Abstract

While much is known about general controls over axon guidance of broad classes of projection neurons (those with long-distance axonal connections), molecular controls over specific axon targeting by distinct neuron subtypes are poorly understood. Corticospinal motor neurons (CSMN) are prototypical and clinically important cerebral cortex projection neurons; they are the brain neurons that degenerate in amyotrophic lateral sclerosis (ALS) and related motor neuron diseases, and their injury is central to the loss of motor function in spinal cord injury. Primary culture of purified immature murine CSMN has been recently established, using either fluorescence-activated cell sorting (FACS) or immunopanning, enabling a previously unattainable level of subtype-specific investigation, but the resulting number of CSMN is quite limiting for standard approaches to study axon guidance. We developed a microfluidic system specifically designed to investigate axon targeting of limited numbers of purified CSMN and other projection neurons in culture. The system contains two chambers for culturing target tissue explants, allowing for biologically revealing axonal growth “choice” experiments. This device will be uniquely enabling for investigation of controls over axon growth and neuronal survival of many types of neurons, particularly those available only in limited numbers.

Introduction

Corticospinal motor neurons (CSMN) control the most precise voluntary movement in mammals. CSMN degenerate centrally in the neurodegenerative “motor neuron” diseases such as amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), and hereditary spastic paraplegia (HSP), and CSMN axonal damage is centrally responsible for the loss of motor function in spinal cord injury. Although the adult mammalian central nervous system (CNS) was classically thought to be incapable of regeneration, more recent work has established that CSMN axon growth inhibition can be at least partially overcome¹⁻⁵. In addition, small numbers of CSMN with long-distance spinal cord projections can be recruited from endogenous progenitors or transplanted as immature neurons developing from appropriate neocortical progenitors⁶⁻⁹. However, attempts at therapeutic regeneration are still limited by an incomplete understanding of mechanisms that control the precise development of these and other neuron subtypes: sequential generation, specification, differentiation, axon guidance, and target selection. A thorough understanding of CSMN axon targeting might enable enhancement of CSMN axon outgrowth and establishment of functional connectivity toward repair of diseased corticospinal circuitry.

In the mouse, CSMN axons descend within the dorsal funiculus of the spinal cord white matter. Leading CSMN axons reach the distal cervical spinal cord ~postnatal day 1 (P1), the distal thoracic cord ~P4, and the distal lumbar cord ~P7¹⁰. These axons arborize among spinal cord interneurons and spinal motor neurons within the segmentally appropriate spinal ventral horn 2-3 days after their arrival at their appropriate level¹¹. CSMN innervate their final targets via interstitial branching from the axon shaft^{12, 13}.

Explant co-culture experiments have partially recapitulated certain aspects of CSMN axon targeting, but a mechanistic understanding of axon growth and survival is still lacking. The presence of one or more diffusible tropic factors was suggested by cortical neurite outgrowth and turning toward explanted spinal cord gray matter^{14, 15}. A role for contact-mediated interactions in segment-specific targeting of CSMN axons was supported by observations of specific ingrowth of cortical axons into segment-appropriate spinal cord gray matter, and repulsion of segment-inappropriate axons by

spinal cord gray matter¹⁶. Field EPSP recordings have demonstrated a preference of forelimb CSMN to form synapses on cervical vs. lumbar spinal cord in explanted slice co-culture¹⁷. Taken together, these findings indicate that molecular mechanisms of CSMN axon targeting involve attractive and repulsive, diffusible and surface-associated, axon guidance cues. A more detailed molecular mechanistic understanding would be advanced by a tissue culture device allowing testing of different combinations of cues, interacting with purified neurons, in three dimensions.

One of our two groups developed approaches to study pure populations of developing CSMN and other projection neuron subtypes in primary culture at a range of critical developmental stages by retrogradely labeling the desired neuron subtype with fluorescent microspheres from their growth cones or axon termini at that developmental stage, then isolating homogeneous populations of specific subtypes of projection neurons by fluorescence-activated cell sorting (FACS)¹⁸⁻²⁰. The resulting pure populations of CSMN in culture retain the *in vivo* cellular and molecular characteristics of CSMN *in vitro*, and have enabled identification and functional analysis of the first peptide controls over their development in a highly-controlled environment²⁰. Unfortunately, the yield of healthy purified CSMN by FACS is approximately 1,000 CSMN per retrogradely labeled mouse brain (out of ~5000 - 6000 in a labeled hemisphere)²⁰. To optimally investigate controls over CSMN axon targeting, it would be highly desirable to have a tissue culture format in which very small numbers of purified CSMN can be studied in each experiment.

Here, we present a new microfluidic device design, based on similar systems developed by our group²¹⁻²³ and others^{24, 25}, for the investigation and quantification of axon outgrowth responses of small numbers of highly purified cortical projection neurons interacting with target tissue explants. This design can confine tissue explants while allowing them to readily communicate with nearby cells via secreted factors. In the device, neurons are co-cultured in a collagen matrix in close proximity to explants of potential target tissue in medium supplemented with defined survival and growth factors. Axons can explore nearby space in three dimensions, as they do *in vivo*. The system contains two chambers for culturing target tissue explants, allowing direct comparisons of potentially differential effects on axon outgrowth. Our device enables

the study of small numbers of cells, higher throughput testing of different targets than in conventional tissue culture, simultaneous testing of diffusible cues, and potentially functionalizing the collagen matrix with surface-associated cues. Using this device, we demonstrate specific targeting of CSMN axons, including axons of purified CSMN, to spinal cord tissue explants, as well as a specific trophic effect of these explants on CSMN axon elongation.

Materials and methods

Device design and fabrication

The device was designed to retain small ($\leq 100 \mu\text{m}$ thick) tissue explants in two separate regions while allowing for rapid, relatively unobstructed interactions with cells seeded in an intervening gel regions. Posts in the device were spaced to help control gel placement while trapping explant tissue, and allowing large regions of direct interaction with growth medium. It was first created in AutoCAD (Autodesk, San Rafael, CA), printed onto a transparency mask using a high-resolution printer (PageWorks, MA), and patterned onto dehydrated master silicon wafers (Wafernet, Inc., San Jose, CA) by standard microfabrication techniques^{26, 27}. Masters were developed using SU8 developer (Microchem Corp., Newton, MA), and treated with trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO) to minimize PDMS adherence. Mixed PDMS (1:10 curing agent: PDMS prepolymer (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, MI)) was degassed, applied to the master wafers, and cured at 80°C for 3 h. Polymerized PDMS devices were peeled off the silicon master, individual devices (30 mm diameter, 1 cm height) cut out, and inlets and outlets for media were bored using a 4 mm dermal biopsy punch. Prior to cell culture, PDMS devices and glass coverslips were autoclaved, oxidized by air plasma for 45 sec, and stored at room temperature for further usage. This surface treatment prevents leaks by forming an irreversible bond between PDMS and cover-slip, post gel or tissue explant seeding.

CSMN labeling, dissociation, purification, and culture

CSMN were retrogradely labeled from C1-C2 under high-resolution ultrasound backscatter microscopic guidance, and then dissociated as previously described^{20, 28, 29}.

Briefly, deeply anaesthetized P2 CD1 mouse pups underwent ultrasound-guided (Visual Sonics, Toronto, ON) injection of green fluorescent Retrobeads IX (Lumafuor, Naples, FL), into the dorsal funiculus between C1 and C2. On P4, brains of injected pups were harvested on ice, and labeled motor cortices were microdissected under a fluorescence dissecting microscope (SMZ- 1500; Nikon) in cold dissociation medium (20 mM glucose, 0.8 mM kynurenic acid, 0.05 mM D(-)-2-amino-5-phosphonovaleric acid (AP5), 50 U/mL penicillin, 0.05 mg/mL streptomycin, 0.9 M Na₂SO₄ and 0.014 M MgCl₂, pH 7.35, and supplemented with B27).

Microdissected motor cortices were enzymatically digested for 15-20 min at 37°C using papain (0.16 mg/L L-cysteine HCl, 12 U/mL papain and 1U/mL DNase I, pH 7.35, prepared in dissociation medium). Enzymatic digestion was blocked by dissociation medium containing 10 mg/mL ovomucoid and 10 mg/mL bovine serum albumin (BSA), and cells were mechanically triturated in OptiMEM (supplemented with 20 mM glucose, 0.4 mM kynurenic acid, 0.025 mM AP5, B27 and BSA). For FACS-purified preparations, dissociated cortical cells were sorted using a FACSVantage/Diva (Becton Dickenson, Franklin Lakes, NJ). Dissociated motor cortical cells were resuspended at $\sim 2 \times 10^5$ /mL in 2 mg/mL collagen type-I (BD Biosciences, San Jose, CA). Collagen solution was prepared by adding collagen stock solution to a mixture of 10 × PBS, 1 M NaOH and tissue culture grade water to obtain a 2 mg/mL solution at pH 7.4. FACS-purified CSMN were resuspended at $\sim 10^4$ /mL in collagen gel, then seeded in devices containing tissue explants, covered with a coverslip, and incubated at 37°C, 100% humidity. Any device containing bubbles or gaps between gel and explant were discarded. Conditioned medium (Neurobasal-A medium supplemented with 0.034 mg/L BSA, 1 mM L-glutamine, 25 U/mL penicillin, 0.025 mg/mL streptomycin, 35 mM glucose and 0.5% B27, conditioned overnight on P2 cortical cultures) was added in the media channels after collagen gel polymerization (~ 20 min), and changed every 24 h. There were no active growth factor gradients across the tissue explants or collagen gel within this device, since the same conditioned media was filled in both the media channels. Furthermore, media channels were filled with equal volumes of media which equalizes pressures and quite effectively prevents the formation of pressure-induced flow across the gel and tissue. All chemicals and reagents were from Sigma Aldrich, and media

from Invitrogen, unless otherwise specified.

Tissue explant preparation

P4 mice were deeply anaesthetized on ice for 6 minutes. The tissue for explant, either the cervical enlargement of the spinal cord or the cerebellum, was exposed and dissected with fine forceps and placed in a small Petri dish filled with ice-cold sterile supplemented OptiMEM. Approximately 100 μm thick axial sections of the cervical spinal cord enlargement or cerebellar hemisphere were cut using a razor blade. Spinal cord sections were then hemisected along the midline, and cerebellar sections were trimmed to size, and stored in ice-cold supplemented OptiMEM until positioning in devices. This protocol for obtaining cervical SC tissue explants has been optimized in our lab and is highly reproducible, which minimizes variations in the axonal targeting data from different experimental batches. These tissue explants retain the architecture and complexity of the spinal cord, specific to their source region, in an *in vitro* culture system.

Immunocytochemistry

Devices were fixed at 48 h by the addition of 2% paraformaldehyde via the media channels, and incubated for 10 min at room temperature. Anti-MAP2 (1:500; Sigma Aldrich, St. Louis, MO), anti-NF (neurofilament heavy chain (NF-H), 1:500; Sigma), and appropriate AlexaFluor secondary antibodies (Molecular Probes, Inc., Eugene, OR) were used. To evaluate CSMN survival, cells were stained using a LIVE/DEAD® Reduced Biohazard Viability Kit (Molecular Probes), fixed, and then imaged using fluorescence microscopy. Phase-contrast and fluorescence microscopy of brain section and dissociated cortical cells shown in Fig. 2 were performed on a Nikon E1000 microscope equipped with an X-Cite 120 illuminator (EXFO), and images were collected with Volocity image analysis software (Improvision, v4.0.1). Phase-contrast and epifluorescence microscopy of cells in devices were performed on a Nikon TE300 microscope with a Hamamatsu camera (Hamamatsu, Shizuoka, Japan) and OpenLab (Improvision, Waltham, MA) image acquisition software.

CSMN morphology assays

All quantification was conducted under blinded conditions using *a priori* criteria. To be identified as CSMN, neurons were required to 1) contain green Retrobeads; 2) exhibit CSMN morphology; and 3) be isolated and lack contact with any other CSMN. Each neuron that fit these criteria (usually 1-2 CSMN per device in dissociated cultures) was photographed and analyzed. Axon length and turning were measured using OpenLab quantification software, and verified with US National Institutes of Health (NIH) imaging software.

Statistical analysis

In this study, at least 10 microfluidic devices were utilized for each experimental condition tested. All statistical analyses were performed using InStat software (v.3.0a, Graphpad) via parametric and/or nonparametric analyses, as appropriate, with a minimum significance level set at $p < 0.01$.

Results

Microfluidic device implementation

We have designed a microfluidic system to investigate controls over cortical projection axon targeting, consisting of a series of channels and gel regions imprinted onto the surface of a PDMS disk and bonded to a coverslip. The system permits axon growth within a three-dimensional matrix, facilitates investigation of interactions between neurons and explanted tissue within the device, and enables real-time imaging of axon outgrowth and targeting. The channels on the bottom surface of the device comprise three adjacent zones: 1) a central neuron soma chamber; 2) two target tissue chambers flanking the central chamber; and 3) two media channels accessible from the top surface of the device. For the axon targeting studies, the trimmed spinal cord or cerebellar tissue explants were manually placed within the target tissue chambers. Then, the cells suspended in a collagen gel were seeded within the soma chamber (~ 1 μ L), which is exposed through lateral openings to the tissue chambers (**Fig. 1a**). The target tissue chambers (150 μ m deep and 1250 μ m in lateral extent) are delineated by square posts arranged in a semi-circle located on both sides of the central neuron soma

chamber (**Fig. 1b**, asterisks), to contain the gel solution by surface tension, and to stabilize the formed gel. The dimensions of posts in this device are 150 μm x 150 μm , spaced to allow media flow and exchange, but close enough to hold the tissue and gel in place. In control cultures with no tissue explants, collagen gel containing cells was filled in all the three zones ($\sim 3 \mu\text{L}$). After seeding the cell-suspended gel alone or in the presence of tissue explants in designated chambers, the bottom surface of the PDMS device and cover-slip were bonded together, closing the channels and completing the device assembly. Media channels (150 μL each side) provide nutrients to the co-culture and serve as vehicles for the addition of supplementary growth factors. This design facilitates efficient gas exchange and waste removal by a direct interface between collagen and culture medium. The interface between the gel and tissue explant allows for passive diffusion of chemotropic substances between the media channels, and establishment of direct contact between axons and explants (**Fig. 1c**). This design minimizes the total volume of gel ($\leq 2 \mu\text{L}$), as well as the number of neurons (~ 5 -10) used per device. Neurite extension is possible in any direction within the gel. This system accommodates two tissue explants, allowing for biologically revealing axonal growth “choice” experiments. The device is well suited to the culture of many types of cortical or other types of neurons and target tissues; we chose to investigate CSMN and spinal cord tissue in our preliminary experiments.

CSMN labeling, culture, and survival

CSMN were retrogradely labeled with green fluorescent latex microspheres at P2 from spinal cord level C1-C2 under high-resolution ultrasound backscatter microscopic guidance, at a stage when CSMN axons are just reaching the cervical spinal cord (**Fig. 2a**). At P4, following retrograde transport of the microspheres, motor cortex was clearly identifiable based upon the presence of green fluorescent CSMN cell bodies (**Fig. 2b**). Motor cortex was microdissected and dissociated to a single cell suspension, enabling identification of individual CSMN based on the presence of green microspheres in their cell bodies (**Fig. 2c**). When cultured as part of unsorted motor cortical dissociates in the microfluidic devices, CSMN (identified by green fluorescent microspheres in their cell bodies) survived for at least 48 h (**Fig. 3**), providing substantial time for extended axon

outgrowth. Image analysis of the LIVE/DEAD® assay cultures (data not shown) indicates excellent CSMN survival (~ 60%) within the microfluidic devices.

Axon targeting in tissue explant co-culture

A major goal of these experiments was to assess the effect of spinal cord tissue explants on CSMN axon growth and targeting. To this end, we cultured dissociated motor cortex with green microsphere-labeled CSMN in the presence of spinal cord tissue explants labeled with crystals of red lipophilic tracer dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), to enable distinct identification of both CSMN and spinal cord (SC) tissue. By 48 h in culture, CSMN extended axons across the explant-gel interface into SC tissue explants, as demonstrated by DiI retrograde labeling from the SC tissue, and by immunocytochemical analysis (**Fig. 4a**). DiI labeling of CSMN was not artifactually due to diffusion; DiI placed in spinal cord tissue explants did not diffuse into the intervening collagen gel, and was not taken up by CSMN axons growing near, but not into, the SC tissue (**Supplemental Fig. 1**).

To assess the potential effects of spinal cord tissue explants on CSMN axon outgrowth, we quantified direction and length of axon outgrowth in the presence or absence of spinal cord explant tissue co-culture. We observed a significant increase in both the turning angle and axon length of CSMN projections when co-cultured with spinal cord tissue explants, compared to control experiments with no explant co-culture (**Fig. 4b-e**).

Purified CSMN axon targeting

To further investigate the role of spinal cord on CSMN axon projections, we co-cultured FACS-purified CSMN with spinal cord tissue explants. In agreement with our findings in motor cortical dissociates, FACS-purified CSMN survived in these devices and projected axons into spinal cord tissue, as demonstrated by DiI retrograde labeling of CSMN axons and cell bodies (**Fig. 5**).

“Choice” experiments between potential target tissues

CSMN project axons toward cervical spinal cord tissues, though the mechanisms

for preference over the neural tissue types remain unclear. These experiments were designed to elucidate CSMN axonal targeting in the presence of two distinct tissue types as possible innervation targets, based on their spatial location in the CNS and inherent ability to attract CSMN axons. To assess the specificity of the attractive axonal growth effects of spinal cord tissue, we co-cultured CSMN between explants of spinal cord tissue and cerebellum as sham CNS tissue (**Fig. 6a-c**). There was a significant increase in CSMN axon outgrowth and directional specificity toward spinal cord vs. cerebellar tissue explants (**Fig. 6d**). These data support roles for specific spinal cord-derived diffusible trophic and chemotropic factors.

Discussion

We report the development of a microfluidic device that enables direct comparison and investigation of the effects of distinct target tissues on axon outgrowth and guidance by purified projection neurons. CSMN survive within these devices long enough to investigate not only survival, but also axon outgrowth and targeting.

A critical design element in the microfluidic device presented here is the precise control on placement of cells and tissues within the 3D milieu, to establish the desired microenvironment. Using traditional tissue culture techniques, it has been challenging to: (i) precisely position small tissue explants and limited numbers of rare cell types in close proximity to mimic physiological 3D microenvironment, and (ii) simultaneously visualize the cellular responses to the diffusing signaling molecules from the tissue explants. Over the past decade, several microfluidic devices have been developed to precisely position and study the neurobiology of small organisms such as *C. elegans*³⁰⁻³², and axonal biology and synapse formation of various neuronal cell types²⁴. The utility of compartmentalized microfluidic platforms to guide growth of axons and dendrites, track movement within axons, identify biochemical composition of axons, and create synapses between distinct neuronal populations, has been recently reviewed by Taylor et al^{24, 25}. Berdichevsky et al. extended existing coculture models of organotypic slices³³⁻³⁵ to microfluidic platforms, by culturing cortex and hippocampal tissue explants within microfluidic compartments interconnected by microchannels, and observed formation of functional synapses with extended axonal networks³⁶. In the present study, we show for

the first time that cervical spinal cord tissue explant induces significant axonal outgrowth and targeting in limited numbers of corticospinal motor neurons, when cocultured in a 3D milieu within a microfluidic device.

A majority of earlier studies on CSMN survival were performed on protein-coated (Ex. Polyornithine, poly-L-lysine) dishes, but not on PDMS, the polymer used for making microfluidic devices in this study. CSMN survival *in vitro* is adversely affected due to both the physical trauma involved in cell isolation – axotomy of exceptionally long axons, tissue dissection, matrix digestion, centrifugation, etc. – and cell biological events including glutamate toxicity and lack of appropriate levels of trophic peptides. Thus, CSMN cultures differ from culture of heterogeneous primary neuron isolates, e.g. from the hippocampus or even the cerebral cortex as pooled populations. Cell viability data are important for co-culture experiments, to understand the responses of these fragile CSMN to tissue explants.

This microfluidic cell culture device offers several advantages for investigating axon targeting controls compared to both conventional tissue culture and other microfluidic systems. Foremost, because of the small volume of the neuron soma chamber, individual experiments using very small numbers of purified neurons can be performed, an important feature in the face of limiting quantities of purified neurons. The low cell density of purified neurons in each device also facilitates assessment of axon length and targeting of individual neurons seeded in the three-dimensional gel. In addition, dual target tissue explant chambers enable effective presentation of a “choice” between potential target tissues, allowing direct comparison of the effects of target-tissue-derived diffusible factors on axon outgrowth and targeting with highly reproducible and symmetric geometry. Finally, because of the relatively small volume of the media channels, even expensive reagents available in very limited quantities can be applied and/or investigated using these devices.

These experiments identify preferential and specific CSMN axon targeting to spinal cord tissue explants co-cultured within the devices. Immunocytochemical analyses and retrograde labeling within the devices confirmed that CSMN project axons toward and into explants of spinal cord tissue, but not cerebellum. In agreement with previous studies^{14, 15}, this provides evidence for diffusible attractants that act at a

distance between spinal cord and CSMN axons. Developmentally, CSMN project their axons prior to much of the cerebellum being formed, and their axonal trajectory does not pass through or toward the cerebellum. These are the primary reasons why we choose cerebellar explants as “sham (control) tissue” for these studies, to investigate the specificity of CSMN targeting toward cervical spinal cord tissue explants when potentially receiving signals from two different types of CNS tissue. Thus, these studies are comparative in nature, and focus on the relative effects of spinal cord and cerebellar tissue.

In conventional tissue explant cultures, CSMN exist in a complex microenvironment surrounded by ECM proteins and various neural and glial cell types, making it difficult to discern their response to external insults from natural variation. Therefore, pure populations of CSMN were isolated and studied in these experiments to achieve a better understanding of their axonal biology *in vitro*. CSMN targeted axons to spinal cord tissue explants, whether the CSMN were cultured as part of motor cortical dissociates or in their FACS-purified form. This is the first analysis of interactions between purified CSMN and any target tissue. The outcomes from such experiments separate multiple variables and reduce the analysis to diffusible substances. Such information might enable peptide or small molecule approaches toward growth and directional control of CSMN axons.

These results also identify a significant and specific increase in CSMN axonal outgrowth in the presence of spinal cord tissue explants. This effect was specific to spinal cord, and was not observed in cerebellar tissue explant co-culture. We have previously demonstrated that insulin-like growth factor-1 (IGF-I) specifically activates and enhances CSMN axon outgrowth²⁰. The increase in axon outgrowth observed in the current study might represent combined effects of not yet identified spinal cord-derived growth factors, possibly in combination with spinal cord-derived IGF-I.

We recently demonstrated the utility of this device in understanding cancer cell migration through 3D gels under flow conditions²¹, and given the robustness and reproducibility of these assays, we anticipate further applications of this microfluidic platform in studying angiogenesis and wound healing. This device could further be used to identify and characterize diffusible and surface-associated molecules controlling

CSMN axon targeting, including controls over spinal cord segmental specificity. It could also be used to identify molecular controls over CSMN survival, and the importance of intermediate and final targets on survival. Our device permits real-time imaging of axon outgrowth and targeting. This device enables a range of approaches to increasingly specifically investigate axon targeting of distinct projection neuron subtypes, toward identification of molecular controls over development, maturation, and function of neuronal circuitry.

Conclusions

A microfluidic culture device was designed, developed and implemented to investigate and compare the effects of distinct target tissues on axon outgrowth and guidance by projection neurons. The device enables individual experiments using very small numbers of purified or enriched neurons, and has enabled the first analysis of interactions between purified corticospinal motor neurons and any target tissue. We used it to demonstrate preferential and specific CSMN axon targeting to spinal cord tissue explants.

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Figure Legends

Figure 1. The microfluidic device provides cells access to growth medium and target tissue. (a) Schematic of the device showing media channels (arrows), tissue wells (asterisks), and soma well (arrowhead). (b) Detail of (a) showing media channels (arrows), tissue wells (asterisks), and soma well (arrowhead). (c) Bright field image of dissociated motor cortical cells (arrowheads) and spinal cord tissue explant (asterisk) seeded in a device. Scale bars, 1 mm.

Figure 2. Injection and retrograde transport from cervical spinal cord specifically labels CSMN. (a) Ultrasound-guided microinjection of green fluorescent microspheres into the cervical spinal cord. Injection is immediately lateral to the midline in the dorsal funiculus. The dorsal/ventral axis is shown (D, V). (b) P4 brain sectioned coronally showing CSMN within layer V of the left hemisphere of neocortex labeled after retrograde transport from the contralateral C1-2 dorsal funiculus. The inset shows magnification of the area indicated. (c) Dissociated cortical cells cultured on poly-L-lysine (PLL) coated coverslips, showing green microsphere labeling of one CSMN (arrowhead).

Figure 3. CSMN in motor cortical dissociate culture survive in devices. (a-d) Survival of CSMN (arrowheads) labeled with green microspheres at 12 h (a-a'''), 24 h (b-b'''), 36 h (c-c'''), and 48 h (d-d'''). Green fluorescent microspheres are shown labeling CSMN (a', b', c', d'). Dead cells labeled by ethidium homodimer are shown in red (a'', b'', c'', d''). Device support posts are labeled in (a, b). The inset in (a) schematizes the region (red box) expanded in (a). Scale bars, 100 μm . Axonal tracings have been included to aid visualization.

Figure 4. CSMN axon outgrowth is directed toward spinal cord tissue explants. (a) Merged fluorescent micrograph showing retrograde Dil labeling from a spinal cord tissue explant (asterisk) in both the axon (arrowheads) and soma (arrow) of a CSMN pre-labeled *in vivo* with green fluorescent microspheres, and co-stained for neurofilament in green. A device support post is outlined (dashed line). (b, c) Overlaid camera lucida images of axons of multiple CSMN cultured in the absence (b) or

presence (c) of spinal cord tissue explants. With no explant co-culture (b), CSMN axons grew equally in all directions. In striking contrast, in spinal cord tissue explant co-culture (c), the majority of CSMN axons grew toward the tissue (direction toward tissue indicated by arrow). (d) Quantification of the direction of axon growth shows significant CSMN axon growth toward spinal cord tissue ($p < 0.001$). (e) Axon length is significantly increased in the presence of spinal cord tissue explants ($p < 0.001$). Quantification performed at 48 h *in vitro*. Scale bars, 100 μm .

Figure 5. FACS-purified CSMN survive and project axons into spinal cord tissue explants. (a) Green fluorescence micrograph showing microspheres in the soma (arrow) of a CSMN, and neurofilament staining along its axon (arrowheads). (b) Dil placed in spinal cord tissue explant (asterisk) is taken up by the CSMN axon (arrowheads) growing into the explant tissue. (c) Merged image showing Dil in the axon (arrowheads) and soma (arrow) of the CSMN. Image taken at 48 h *in vitro*. Scale bars, 100 μm .

Figure 6. CSMN axons grow specifically toward spinal cord tissue explants. (a) Cells loaded in the device with tissue explants from spinal cord and cerebellum. (b) In the vicinity of spinal cord explant tissue, CSMN (arrows) axons project toward the explant. (c) Cerebellar explants have no effect on the length or direction of CSMN (arrow) axon outgrowth. (d) Overlaid camera lucida images of CSMN axons near spinal cord or cerebellar explant. Scale bars, 100 μm .

Supplemental Figure 1. FACS-purified CSMN take up Dil only by direct contact with spinal cord tissue and not by diffusion. (a) Green fluorescence micrograph showing microspheres in the soma (arrow) of a CSMN, and neurofilament staining along its axon (arrowheads). (b) Dil placed in spinal cord tissue explant (asterisk) is not taken up by the CSMN axon growing near, but not into, the tissue. (c) Merged image showing the absence of Dil from the axon (arrowheads) and soma (arrow) of the CSMN. Image obtained at 48 h *in vitro*. Scale bars, 100 μm .

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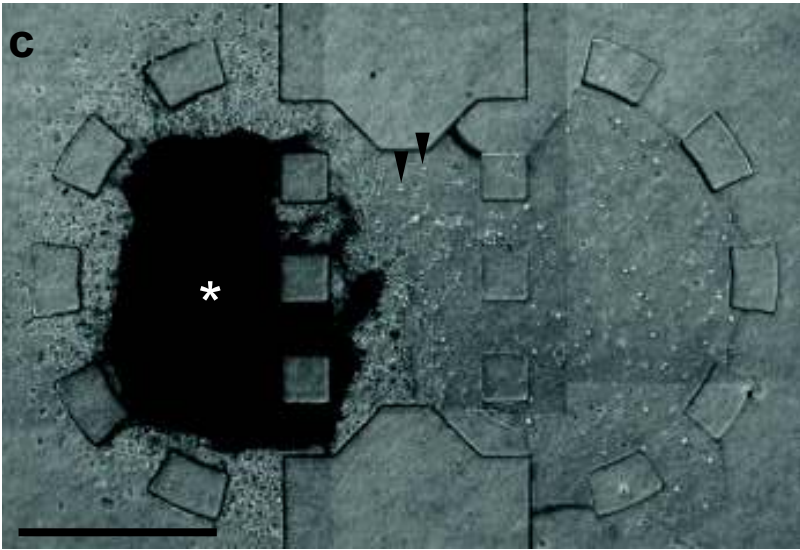
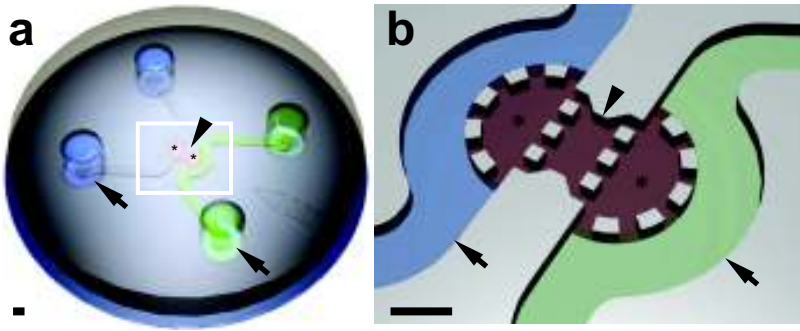
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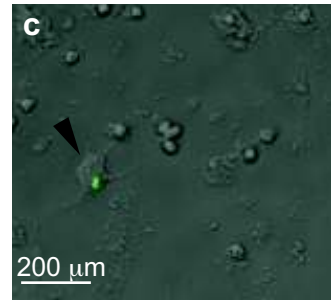
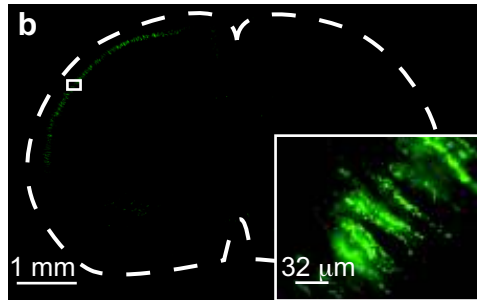
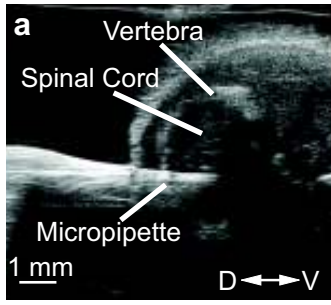
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Type of file: figure

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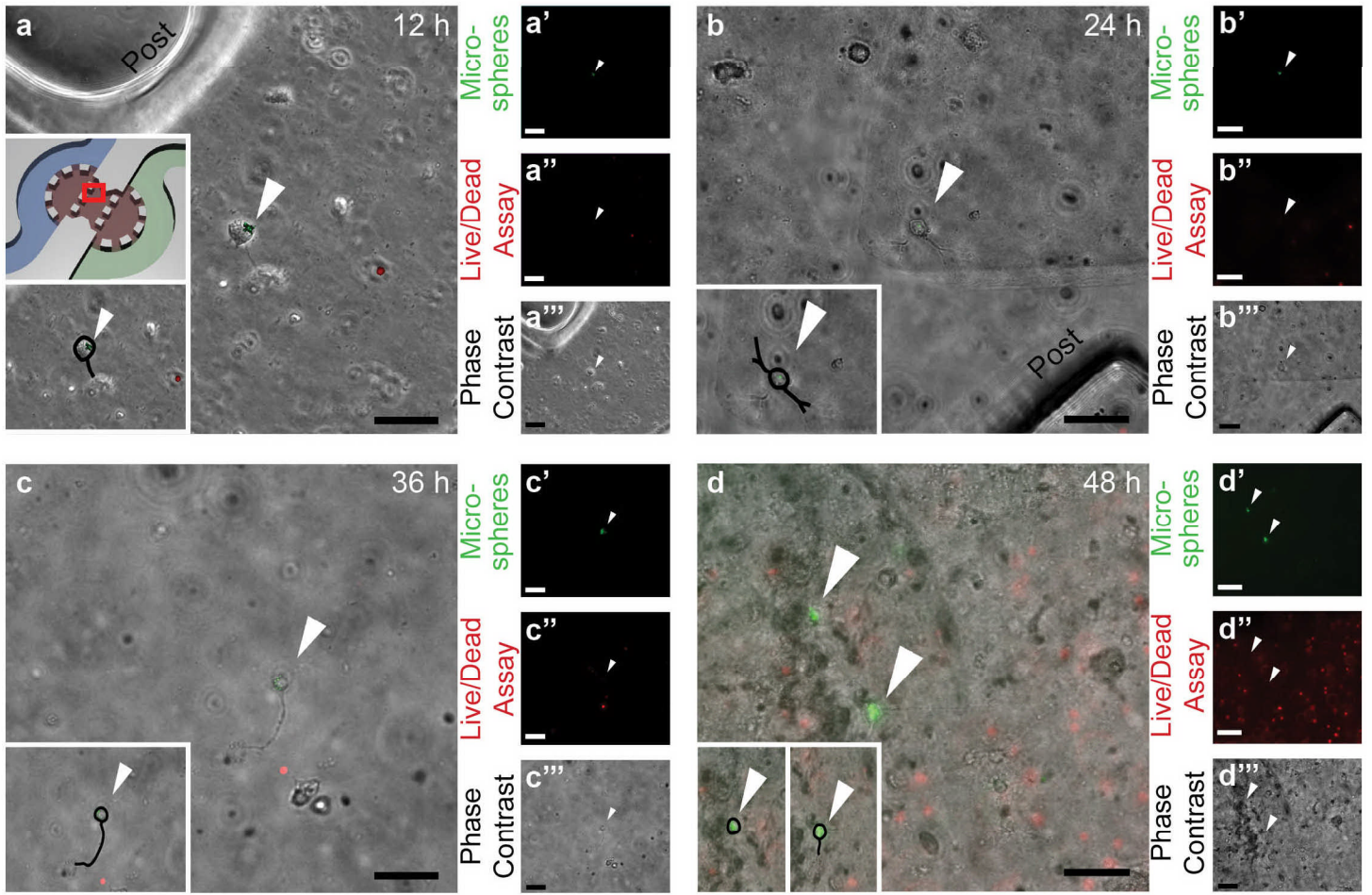
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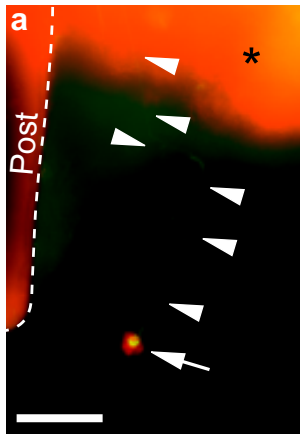
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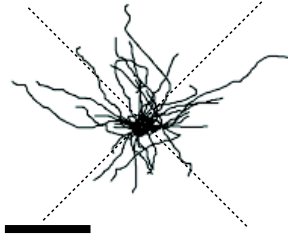
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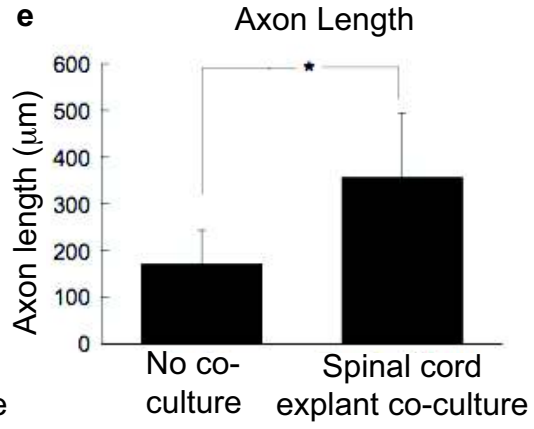
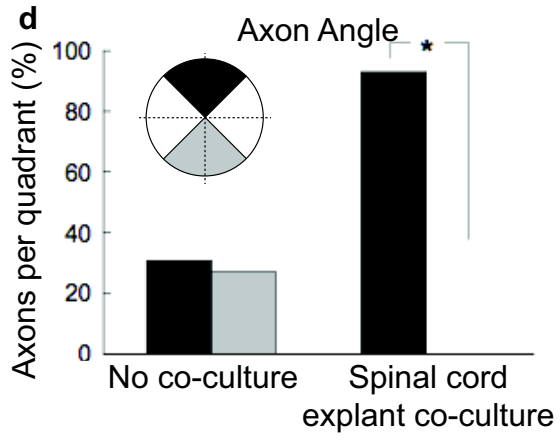
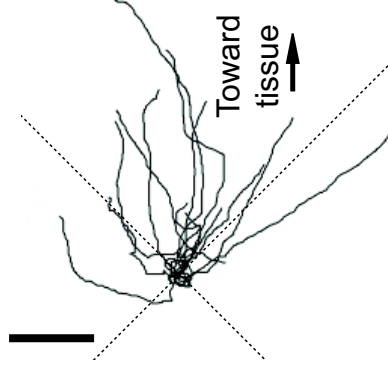
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b No co-culture



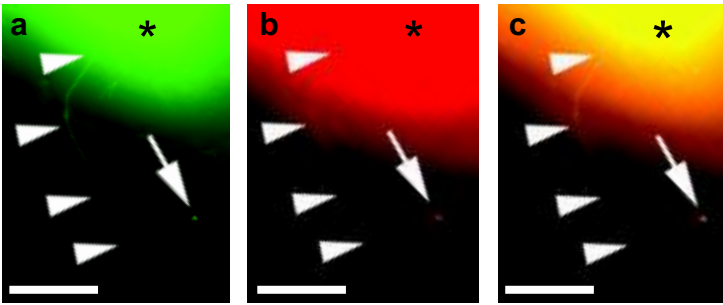
c Spinal cord explant co-culture



Type of file: figure

Label: Figure 5

Filename: Figure 5 Macklis.eps



Microspheres +
Neurofilament

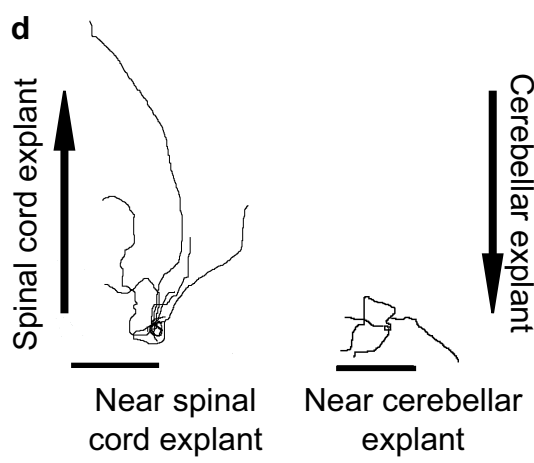
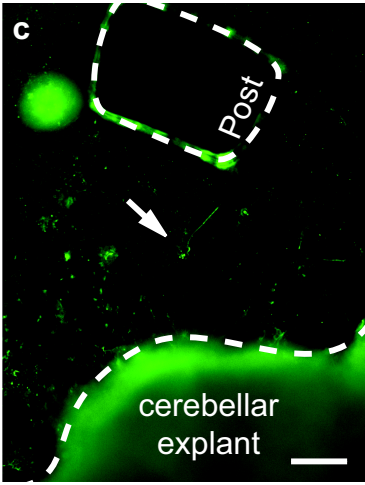
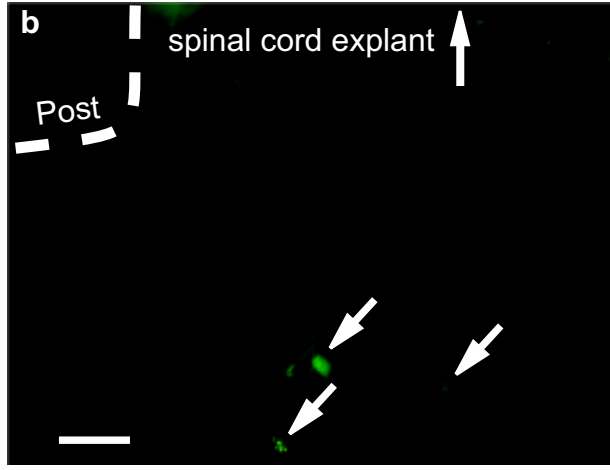
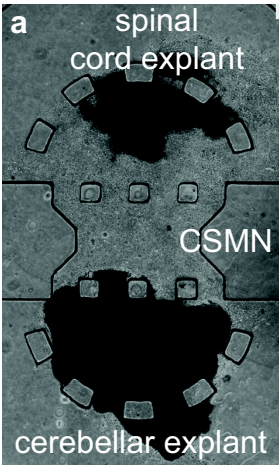
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Merge

Type of file: figure

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