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TISSUE ENGINEERING THE MOTONEURON TO MUSCLE SEGMENT OF THE STRETCH
REFLEX ARC CIRCUIT UTILIZING MICRO-FABRICATION, INTERFACE DESIGN AND DEFINED
MEDIUM FORMULATION

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biomedical Sciences
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
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Major Professor: James J Hickman

ABSTRACT

The stretch reflex circuit is one of the most primitive circuits of mammalian system and serves mainly to control the length of the muscle. It consists of four elements: the stretch sensor (muscle spindle/ intrafusal fiber lie parallel between extrafusal, contractile musculature), extrafusal muscle fiber, sensory neuron and motoneuron. The basic principle of the stretch reflex arc circuit is as follows: whenever there is a sudden stretch in a muscle, it needs to compensate back to its original length so as to prevent any kind of injury. It performs this compensation process using a simple negative feed back circuit called the stretch reflex arc. Any form of stretch in a muscle activates the stretch sensors (muscle spindle/ intrafusal fiber) lying deep in each muscle. After the stretch sensors get activated, it sends a train of signals to the spinal cord through the sensory neurons. The sensory neurons relay this information to the motoneuron. The motoneuron performs the necessary information processing and sends the message to the extrafusal fibers so as to compensate for the sudden stretch action. The motoneuron conveys this message to the extrafusal fibers by communicating through the special synaptic junctions called neuromuscular junctions. Based on this information, the extrafusal fibers act accordingly so as to counter the effect of sudden stretch. This is also called the monosynaptic stretch reflex that involves a single synapse between a sensory neuron and a motoneuron. To date studying these stretch reflex circuits is only feasible in animal models. Almost no effort has been made to tissue engineer such circuits for a better understanding of the complex development and repair processes of the stretch reflex circuit formation. The long-term goal of this research is to tissue engineer a cellular prototype of the entire

stretch reflex circuit. The specific theme of this dissertation research was to tissue engineer the motoneuron to muscle segment of the stretch reflex arc circuit utilizing micro-fabrication, interface design and defined medium formulations. In order to address this central theme, the following hypothesis has been proposed. The first part of the hypothesis is that microfabrication technology, interface design and defined medium formulations can be effectively combined to tissue engineer the motoneuron to muscle segment of the stretch reflex arc. The second part of the hypothesis is that different growth factors, hormones, nanoparticles, neurotransmitters and synthetic substrate can be optimally utilized to regenerate the adult mammalian spinal cord neurons so as to replace the embryonic motoneurons in the stretch reflex tissue engineered construct with adult motoneurons. In this body of work, the different tissue engineering strategies and technologies have been addressed to enable the recreation of a *in vitro* cellular prototype of the stretch reflex circuit with special emphasis on building the motoneuron to muscle segment of the circuit. In order to recreate the motoneuron to muscle segment of the stretch reflex arc, a successful methodology to tissue engineer skeletal muscle and motoneuron was essential. Hence the recreation of the motoneuron to muscle segment of the stretch reflex circuit was achieved in two parts. In the part 1 (Chapters 2-5), the challenges in skeletal muscle tissue engineering were examined. In part 2 (Chapters 6-7), apart from tissue engineering the motoneuron to muscle segment, the real time synaptic activity between motoneuron and muscle segment were studied using extensive video recordings. In part 3 (Chapters 8-10), an innovative attempt had been made to tissue engineer the adult mammalian spinal cord neurons so that in future this technology could utilized to replace the

embryonic neurons used in the stretch reflex circuit with adult neurons. The advantage of using adult neurons is that it provides a powerful tool to study older neurons since these neurons are more prone to age related changes, neurodegenerative disorders and injuries. This study has successfully demonstrated the recreation of the motoneuron to muscle segment of the stretch reflex arc and further demonstrated the successful tissue engineering strategies to grow adult mammalian spinal cord neurons. The different cell culture technologies developed in these studies could be used as powerful tools in nerve-muscle tissue engineering, neuro-prosthetic devices and in regenerative medicine.

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TABLE OF CONTENTS

LIST OF FIGURES.....	ix
CHAPTER 1: GENERAL INTRODUCTION.....	1
References	14
CHAPTER 2: DEVELOPMENT OF A NOVEL SERUM-FREE CELL CULTURE MODEL OF SKELETAL MUSCLE DIFFERENTIATION BY SYSTEMATIC INVESTIGATION OF THE ROLE OF DIFFERENT GROWTH FACTORS IN MYOTUBE FORMATION	26
Introduction	26
Methods and Materials	29
Results and Discussion	35
References	54
CHAPTER 3: A DEFINED SYSTEM TO ALLOW SKELETAL MUSCLE DIFFERENTIATION AND SUBSEQUENT INTEGRATION WITH SILICON MICROSTRUCTURES. BIOMATERIALS. 2006 AUG;27(24):4374-80.....	65
Introduction	65
Methods and Materials	68
Results and Discussion	75
References	87
CHAPTER 4: DIFFERENTIATION OF SKELETAL MUSCLE AND INTEGRATION OF MYOTUBES WITH SILICON MICROSTRUCTURES USING SERUM-FREE MEDIUM AND A SYNTHETIC SILANE.NATURE PROTOCOLS 2007;2(7):1795-801	92
Introduction	92
Materials and Methods	97
Results and Discussion	118
References	127
CHAPTER 5: SKELETAL MUSCLE TISSUE ENGINEERING: AN IMPROVED MODEL PROMOTING LONG TERM SURVIVAL OF MYOTUBES, STRUCTURAL DEVELOPMENT OF EXCITATION-CONTRACTION (E-C) COUPLING APPARATUS AND NEONATAL MYOSIN HEAVY CHAIN (NHMC) EXPRESSION.....	129
Introduction	129
Methods and Materials	133
Results and Discussion	141
References	164

CHAPTER 6: EMBRYONIC MOTONEURON-SKELETAL MUSCLE CO-CULTURE IN A DEFINED SYSTEM. NEUROSCIENCE. 2007 MAY 11;146(2):481-8.....	183
Introduction.....	183
Methods and Materials	184
Plating the embryonic.....	186
Results and Discussion	192
References	215
CHAPTER 7: TISSUE ENGINEERING THE NEUROMUSCULAR JUNCTION: A DEFINED MODEL SYSTEM	219
Introduction.....	219
Methods and Materials	222
Results and Discussion	230
References	261
CHAPTER 8: ADULT RAT SPINAL CORD CULTURE ON AN ORGANOSILANE SURFACE IN A NOVEL SERUM-FREE MEDIUM. IN VITRO CELL DEV BIOL ANIM. 2005 NOV-DEC;41(10):343-8.).....	278
Introduction.....	278
Methods and Materials	280
Result and Discussion	286
References	296
CHAPTER 9: AUTO-CATALYTIC CERIA NANOPARTICLES OFFER NEUROPROTECTION TO ADULT RAT SPINAL CORD NEURONS. BIOMATERIALS. 2007 APR;28(10):1918-25).....	303
Introduction.....	303
Methods and Materials	305
Results and Discussion	309
References	328
CHAPTER 10: TEMPORAL NEUROTRANSMITTER CONDITIONING RESTORES THE FUNCTIONAL ACTIVITY OF ADULT SPINAL-CORD NEURONS IN LONG-TERM CULTURE. EXP NEUROL. 2008 JAN;209(1):171-80	333
Introduction.....	333
Methods and Materials	335
Results and Discussion	344
References	367
CHAPTER 11: GENERAL DISCUSSION.....	372
References	381
APPENDIX: COPYRIGHT PERMISSION LETTERS	384

LIST OF FIGURES

Figure 1. Interdisciplinary roots of tissue engineering	3
Figure 2. Stretch reflex arc circuit.....	7
Figure 3. A cantilever-based bio-MEMS device to measure communication between a motoneuron and myotube. Each component of the system has been marked with a number (1-14). The detail of each part of the device has been explained below: 1. Custom cantilever arrays; 2. Detailed layout of pattern on cantilever array; 3. Individual cantilever; 4. Site for motoneuron plating on patterned substrate; 5. Site for sensory neuron plating on patterned substrate; 6. Barrier between motoneuron and sensory neuron plating sites; 7. Site for skeletal muscle myotube formation; 8. Putative neuromuscular junction site between motoneuron and skeletal muscle; 9. Deflection of cantilever due to muscle contraction after receiving signal from motoneuron; 10. Source for laser beam; 11. Laser beam falling on skeletal muscle on top of cantilever; 12. Reflecting laser beam; 13. Reflecting laser beam changes angle due to bending of cantilever caused by contracting myotubes; 14. Photo-detector measuring the change in angle θ	10
Figure 4. Flow chart showing the technique to grow robust myotubes and muscle-nerve cocultures: The formulation IX and the medium change protocol which is most optimal for myotube and coculture growth.	32
Figure 5. Different morphologies of myotubes stained with embryonic myosin heavy chain antibodies (Red) and clustering of acetylcholine receptor (Green) on the membrane surface of myotubes. Scale bar was 50 μm . A. Chain like morphology of myotubes. B. Branched morphology of myotubes.C. Spindle shaped morphology of a single myotube.D. Cylinder shaped morphology of a single myotube.E-H. Different morphologies of myotubes showing	

the clustering of acetylcholine receptors (Green) on the membrane surface of different myotubes.....	45
Figure 6. Coculture of skeletal muscle* sensory neurons (DRG)* spinal cord neurons: All the neurons were stained with antibody against neurofilament-M (150 KD) (Red) and the myotubes were stained with antibody against embryonic myosin heavy chain (F 1.652) (Green). Scale bar for was 75 μ m. A. The large sensory neurons (DRG) were seen stained with NF 150 (Red). The neurons are shown with white arrow. The myotubes stained with F1.652 (Green) were seen in the same field. B. The neuron process in red were seen running parallel with the myotubes (Green).C. The multipolar motoneuron was seen in close proximity with the myotubes. In the frame we also observed multiple large sensory neurons. All the neurons were pointed out bu white arrows. D. A bundle of multiple processes of neurons crossed the striated, branched myotube. E. A single multipolar motoneurons was observed to form a wrapping around the mytotube.F. Neuron processes formed wrapping on the myotubes.....	48
Figure 7. SEM micrograph of the fabricated cantilever.	70
Figure 8. Representative pictures of myotubes formed in our defined system at day 4 and 5. A: E18 non-purified culture. Myotubes were formed on the top of a monolayer of other cell types. B: Myotubes in E18 purified cultures. C, D: Multinucleated myotubes in E17 cultures. E: Myotubes immunostained for myosin heavy chain. F: Myotubes immunostained for α -actin. A, B, C, D: Phase contrast 40x, scalebar: 25 μ m. E: Confocal 40x, scalebar: 75 μ m. F: Confocal 63x, scalebar: 40 μ m.....	81
Figure 9. Representative electrophysiological recordings obtained from 4-day-old myotubes. (A) Voltage-clamp experiments indicated that myotubes formed on a DETA surface in the serum-free medium formulation expressed functional voltage-dependent sodium and potassium channels. (B) Current-clamp mode depolarization with evoked action potentials, which were associated with visible contractions.....	83

Figure 10. Myotubes forming on fabricated microcantilevers in serum-free medium in two separate experiments (middle and right). <i>Note:</i> the myotubes generally lined up with the long axis of the cantilever.	85
Figure 11. General outline of the entire procedure.	95
Figure 12. Outline of microcantilever manufacture procedures.	103
Figure 13. SEM micrographs of microcantilever array. (a) Top down view of cantilevers. (b) 45° angle view of cantilever array.	104
Figure 14. Defined system for growing skeletal muscle. Outline of the cell culture process.	110
Figure 15. Phase pictures of myotubes. A. Phase picture of 7 day old myotubes. B. Phase picture of a 7 day old myotube with a patch-clamp electrode on its surface for electrophysiological studies.	119
Figure 16. Immunostaining pictures of myotubes. A. Alpha-actin immunostained picture of 7 day old myotubes B. Embryonic myosin heavy chain stained picture of a 7 day old myotube.	121
Figure 17. 7 days old pictures of the myotubes growing on the cantilevers. Myocytes are aligning along the long axis of the cantilever and forming contracting myotubes.	123
Figure 18. Electrophysiological recordings. Panel A indicates the representative voltage clamp traces obtained after patching a 7 day old myotube. Panel B showing the representative current clamp traces obtained after patching a 7 day old myotube.	126
Figure 19. Schematic diagram showing the culture protocol.	135
Figure 20. A, B, C and D: Phase pictures of 50-day-old myotubes in culture. Red arrows show characteristic striations in most of the myotubes. Scale bar: 75 micron.	143
Figure 21. Myotubes stained with antibodies against embryonic myosin heavy chain (F 1.652) proteins at day 50. Scale bar: 75 micron. A. Panel showing phase + fluorescent picture of the myotubes. B. Panel A observed only under fluorescent light. C. Panel showing phase + fluorescent picture of the myotubes. D. Panel C observed only under fluorescent light. E. Panel showing phase + fluorescent picture of the myotubes. F. Panel E observed only under	

fluorescent light. G. Panel showing phase + fluorescent picture of the myotubes. H. Panel G observed only in fluorescent light. White arrows show the striations in panels B, E, F, G and H.....146

Figure 22. Myotubes immunostained with neonatal myosin heavy chain (N3.36) and alpha-bungarotoxin at day 50. Scale bar: 75 micron. A. Phase picture of 2 myotubes shown by the white arrows. B. Both myotubes shown in phase (Fig A) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining. C. Only one myotube out of the two seen in Fig A stained for N3.36. D. Double stained picture of the Fig A with alpha-bungarotoxin and N3.36. E. Phase picture of 6 myotubes, shown by white arrows. F. All the myotubes shown in phase (Fig E) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining. G. None of the myotubes in Fig E stained for N3.36. H, I, J. Differential staining of the myotubes with N3.36. K, L, M. Differential staining of the myotubes with N3.36.149

Figure 23. Ryanodine receptor and DHPR receptor clustering in 30-day-old skeletal muscle culture. Scale bar 75 micron. A. Phase and fluorescent-labeled picture of the myotubes. B. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes shown in panel A. C. Ryanodine receptor (Green) on the myotubes shown in panel A. D. DHPR receptors on the myotubes shown in panel A. E. Phase and fluorescent labeled picture of the myotubes. F. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes (Panel E). G. Ryanodine receptor (Green) on the myotubes (Panel E). H. DHPR receptors on the myotubes (Panel E). I. Phase and fluorescent-labeled picture of the myotubes. J. Phase and fluorescent-labeled picture of the myotubes. K and L. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes (Panel I) on three different planes. White arrows show the striations and the receptor clustering along the striations. ..153

Figure 24. Ryanodine receptor and DHPR receptor clustering in 100-day-old skeletal muscle culture. Scale bar: 75 micron. A. Phase and fluorescent-labeled picture of the myotubes. B.

Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes (Panel A). C. Ryanodine receptor (Green) on the myotubes (Panel A). D. DHPR receptors on the myotubes (Panel A). E and F. Same panels at different plane showing the merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes.....	157
Figure 25. Patch clamp electrophysiology of the myotubes. A. Representative voltage clamp trace obtained after patching a 48-day-old myotube in culture (n>20) B. Representative current clamp trace of the same myotube, which a voltage clamp trace had been obtained (n>20). Inset showing the picture of patched myotubes. (n= number of myotubes which were patched during the course of study).....	160
Figure 26. Defined model system for the embryonic motoneuron-muscle co-culture.....	187
Figure 27. Immunostaining of the myotubes and the motoneurons separately in co-culture. A,B. Myotube morphology and immunocytochemistry in co-culture was done using the fetal myosin heavy chain antibody and appear green. Myotubes and characteristic nuclei can be easily seen. C, Motoneuron morphology in co-culture was visualized using phase contrast microscopy and the cell soma and processes are easily distinguished. D, Motoneuron immunocytochemistry using the NF-150 antibody. Cell soma and processes appear red. Scale bars: 20 microns in all panels.....	196
Figure 28. Phase contrast and immunocytochemical characterization of myotubes and motoneurons in co-culture. A,B, Myotubes stained for fetal myosin heavy chain (green) and motoneurons stained for neurofilament 150 (red) are shown in proximity to each other with neuronal processes touching the myotubes in two planes (blue arrows). C,D, Phase contrast and confocal images of a myotube (green) innervated by multiple neuronal processes (red) and in proximity to a motoneuron (red). E,F, phase contrast and alpha-bungarotoxin staining of motoneuron-myotube co-culture, where the blue arrow points to the motoneuron, and red arrows and green dot clusters indicate areas of acetylcholine receptor clustering.	199

Figure 29. Immunocytochemical evidence for synapse formation at the NMJ. A. Phase picture of a motoneuron in close proximity to a myotube. The motoneuron was stained with synaptophysin (red). B. The motoneuron labeled with synaptophysin (red) and the myotube labeled with alpha-bungarotoxin (green) was observed at a specific plane in the confocal image. C, D. The process of the motoneuron appears to be synapsing on the myotube to form a NMJ in two different planes as seen by the close proximity of the presynaptic marker, synaptophysin (red), and the postsynaptic acetylcholine receptor clustering was indicated by alpha-bungarotoxin labeling (green). Scale bar is 25 μ201

Figure 30. Figure A and B shows the contaminating glial cells (GFAP positive cells are shown in red) present in the co-culture.....203

Figure 31. Figure A shows the phase picture of the coculture stained with an anti-fibroblast marker. Figure B indicates the absence of a fibroblast in the coculture after staining with an anti-fibroblast marker (green).205

Figure 32.[A]. Electrophysiological properties of a single motoneuron in co-culture. A, voltage clamp recording of the patched motoneuron showing active Na⁺ and K⁺ currents. B, Phase contrast image of patched motoneuron (red arrow) in proximity to a myotube (green arrow). C, Current clamp recording of the patched motoneuron showing a repetitively firing action potential. These recordings are representative of 95% of the neurons present in the culture.[B]. Electrophysiological properties of a single myotube in co-culture. A. Voltage clamp recording of the patched myotube showing active Na⁺ and K⁺ currents. B, Phase contrast image of patched myotube (green arrow) in proximity to a motoneuron (red arrow). C, Current clamp recording of the patched myotube showing a single action potential. These recordings are representative of 80% of the myotubes present in the culture.....208

Figure 33. Figure A and B indicate the phase picture of the embryonic motoneuron and myotube pair forming a NMJ. The myotube has been shown to contract rhythmically in Video 2. After the application of the nicotinic cholinergic antagonist d-tubocurarine, the rhythmic contraction

of the myotube stopped. Video 2 recorded for 1 minute and 54 seconds. The antagonist d-tubocurarine was applied 45 seconds after the recording started. We observed that the rhythmic contraction of the myotube gradually stopped within the next 10 seconds. Although recordings continued for an additional minute, no further contractions of the myotubes were observed.....210

Figure 34. The modified protocol for co-culturing motoneuron and skeletal muscle.....227

Figure 35. A-D: Phase contrast pictures of the motoneurons and skeletal muscle in co-culture between day 12-15. The distinct morphology of motoneuron and the processes were indicated by red arrows. The myotubes were shown by green arrows. The scale bar is 25 μ235

Figure 36. Phase contrast pictures of the co-cultures between day 25-30. A,B: The myotubes showing characteristic striations. C,D: Panels showing myotubes with striations and myotubes without striations. The red arrows indicate the neuron cell body and the processes. The green arrow indicated the myotubes. The scale bar for A, B is 40 μ . The scale bar for C, D is 25 μ240

Figure 37. A,B: Cocultures at day 25 were stained with NF-150 and N3.36. NF-150 (red) stained the large motoneurons and their processes. The motoneurons were shown by white arrows. The myotubes (green) stained for N3.36. The neuronal processes were seen all over the myotubes. The characteristic striations of the myotubes were observed in the pictures. The scale bar was 50 μ244

Figure 38.A-D: Neuromuscular Junction (NMJ) formation between day 30-40. The embryonic motoneuron-skeletal muscle coculture were stained with neonatal myosin heavy chain (N3.36) antibody (Red), alpha-bungarotoxin (post-synaptic marker labeled with green) and synaptophysin (pre-synaptic marker labeled with blue). Colocalization of pre-synaptic and post-synaptic markers indicate the synapse formation at NMJ. A. Phase picture of the myotube showing the alpha-bungarotoxin staining in green. B. Triple stain, showing the close

proximity of alpha-bungarotoxin (green) and synaptophysin (blue) indicating synapse formation at a specific plane and the striations of the muscle were seen in red (NMHC). C-D. Observing the NMJ at two different planes using the confocal microscopy. A much more dense clustering of synaptophysin and alpha-bungarotoxin was observed at these planes.....249

Figure 39. A, B: No NMJs were observed in these striated muscle. Although alpha-bungarotoxin was observed on the surface of the myotubes indicating the clustering of the acetylcholine receptors. But no distinct colocalization of synaptophysin was observed in close proximity of acetylcholine receptor clusters. A. The phase picture of the myotube. B. Immuno-stained picture of the same myotube with alpha-bungarotoxin, N3.36 and synaptophysin. The scale bar for both the pictures is 50 μ252

Figure 40. Formation of NMJ on a myotube which was not positive for N3.36. the cultures was stained with alpha-bungarotoxin, N3.36 and synaptophysin. A. Phase picture showing the different morphologies of myotubes in the co-culture. B-D. NMJ formation was observed on a myotube which was not positive for N3.36. Possibly the myotube on which NMJ was formed was still immature and did not express the neonatal myosin heavy chain (N3.36).....257

Figure 41. (a) Structure of a N-1(3-[trimethoxysilyl]propyl)-diethylenetriamine (DETA) molecule. Cartoon showing the DETA coating on a glass coverslip. (b) Isolated fragment of adult rat spinal cord (left). Major band of spinal cord cells obtained after optiprep gradient centrifugation (right).....281

Figure 42. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, CAM 200) (data not shown) and by X-ray photoelectron spectroscopy (XPS; Kratos Axis 165) by monitoring the N 1 s peak. This figure shows an XPS survey scan of the N-1(3-[trimethoxysilyl]propyl)-diethylenetriamine monolayer.287

Figure 43. a. Phase contrast picture of neuronal and glial cells in the adult spinal cord culture (day 6 *in vitro*), Scale bar: 50 micron. b. Phase contrast picture of neuronal and glial cells in the adult spinal cord culture (day 25 *in vitro*), Scale bar: 50 micron.c. Immunostaining with anti-

neurofilament 150, a neuron specific marker (red) and anti-GFAP, a glial cell marker (green) (day 25 day *in vitro*).d. Second neuronal specific marker for anti-NSE (red) (day 15 *in vitro*). e. Anti-Islet-1 staining of cells that exhibited a neuronal morphology (day 25 *in vitro*). The nucleus is brightly stained with Islet-1 (green) which is a putative motoneuron marker. f. Representative voltage clamp recordings obtained from neuronal cells on day 10 *in vitro*. Voltage dependent ionic currents were evoked by voltage steps from -40 to +20 mV.....290

Figure 44. Synthesis and Characterization of Cerium oxide Nanoparticles. (a) Outline of the microemulsion technique to synthesize the nano-Ceria (b) XPS analysis of synthesized Cerium oxide nanoparticles showing the presence of both Ce³⁺ and Ce⁴⁺ valence states (c) HRTEM image of the synthesized nanoparticles showing uniform particle size in the range of 3-5 nm.311

Figure 45. Adult Rat Spinal Cord Culture (a) Isolation of adult rat spinal cord cells from the whole cord (b) Development of serum-free culture medium using various growth factors (c) Surface modification of the glass cover slips for cell culture.314

Figure 46. Live-Dead Assay, Neuron-Glial Cell Assay and Electrophysiological Studies of Control and nano-Ceria Treated Cultures of Adult Rat Spinal Cord (a) Live-dead cell assays indicated that nano-Ceria treated cultures had significantly higher cell survival and significantly less cell death at day 15 and day 30 in culture as compared to the control cultures (b) Neuron-glial cell assays indicated that a significantly high neuronal survival in treated cultures at day 15 and day 30 as compared to the control cultures. (Data are presented as Mean ± SE, n = 6; * p < 0.05; ** p < 0.01).317

Figure 47. Voltage-clamp recording from a treated culture at day 30 (left) Current clamp recording indicating a single action potential in a nano-Ceria treated culture at day 30 (right).....319

Figure 48. Schematic Detailing the Proposed Regenerative Properties of nano-Ceria and probable mechanism of Cerium oxide nanoparticles' free radical scavenging property and auto-catalytic behavior.....321

Figure 49. UV-visible study of Cerium Oxide nanoparticles treated with hydrogen peroxide at different time intervals.323

Figure 50. Results After Hydrogen Peroxide-Induced Oxidative Injury in Control and Treated Cultures of Adult Rat Spinal Cord at day 30. Live-dead cell assay after hydrogen peroxide treatment indicates that nano-Ceria treated cultures had a significantly higher number of surviving cells as compared to the control (Data are presented as Mean \pm SE, n = 6; * p < 0.05; ** p < 0.01).326

Figure 51. Outline of the defined culture system to study the regeneration of adult mammalian spinal cord neurons.336

Figure 52. Immunocytochemical evidence of the early events during the initiation of the regeneration process utilizing nestin and neurofilament-150. Upper panel. The regeneration process was initiated during the first 24 hours of cell plating and the live/dead assay indicates the majority of the plated cells are alive. Lower panel. Early regeneration events are characterized by co-expression of the nestin and neurofilament 150 proteins by most neurons between day 1-3. By day 4, the neurons only express neurofilament-150 and other neuron specific markers, as the nestin expression was lost by day 4.....346

Figure 53. Immunostained cultures at day 35 utilizing different neuron specific antibodies. A. Phase coupled with fluorescence micrograph showing neurons stained with ISLET-1 antibody (a putative motoneuron marker). B. Fluorescent staining of the ISLET-1 positive cells shown in figure A. C. Neurons stained with MO-1 antibody (a putative motoneuron marker). D. Neurons stained with ChAT antibody (a putative motoneuron marker). E. Neurons double-stained with MAP 2a and b and NF 150 antibodies. F. Neurons double-stained with synaptophysin and NF 150 antibodies. G. Neurons stained with NF 150 antibody. H. Neurons stained with MAP 2, a and b antibody.....348

Figure 54. Representative phase-contrast pictures of the cells which were used to quantify the electrical properties. A and B. Phase pictures of the neurons in control culture at day 44. C.

Phase pictures of the neurons after glutamate treatment at day 37 (G37). D. Phase pictures of the neurons after glutamate treatment at day 44 (G44). E. Phase pictures of the neurons after serotonin treatment at day 37 (S37). F. Phase pictures of the neurons after serotonin treatment at day 44 (S44). G. Phase pictures of the neurons after acetylcholine chloride treatment at day 37 (A37). H. Phase pictures of the neurons after acetylcholine chloride treatment at day 44 (A44). I. Phase pictures of the neurons after glutamate+serotonin treatment at day 37 (GS37). J and K. Phase pictures of the neurons after glutamate+serotonin treatment at day 44 (GS44). L, M, N and O. Phase pictures of the neurons after glutamate+serotonin followed by acetylcholine chloride treatment at day 44 (GSA44).350

Figure 55. Electrophysiological recordings from glutamate+serotonin→acetylcholine chloride (GSA44) treated cultures. A. Scheme for single neurotransmitters application. B. Scheme for multiple neurotransmitters application.356

Figure 56. A. Representative trace for voltage and current clamp of a neuron firing a single action potential after multiple neurotransmitter applications at day 44. B. Representative trace for voltage and current clamp recordings of a double action potential firing neuron after multiple neurotransmitter applications at day 44. C. Representative trace for voltage and current clamp recordings of a neuron firing multiple action potentials after multiple neurotransmitter applications at day 44.361

LIST OF TABLES

Table 1. Development of chemically defined serum-free medium by systematically adding individual growth factors in the culture. Each of these experimental trials had been carried out atleast 6 times ($n \geq 6$).	37
Table 2: Composition of novel serum-free medium for a 500 ml sample.....	42
Table 3: Composition of the Serum-Free Medium for a 500ml Sample.....	78
Table 4: Troubleshooting	117
Table 5: Composition of medium 1.	137
Table 6: Composition of medium 2	138
Table 7: Serum-free co-culture medium composition.....	194
Table 8: Composition of medium 1.	225
Table 9: Composition of medium 2	226
Table 10: Composition of 500 ml serum-free medium.....	283
Table 11: Composition of the serum-free medium.	339
Table 12: Comparison of the total number of cells patched and the number of cells which exhibited APs in control (C37, C44), glutamate treated (G37, G44), serotonin treated (S37, S44), acetylcholine chloride treated (A37, A44), glutamate+serotonin treated (GS37, GS44), and glutamate+serotonin→ acetylcholine chloride treated (GSA44). 37 and 44 indicates 7 and 14 days after culturing the cells in the presence of neurotransmitters respectively. Percentages are indicated in parentheses.	352
Table 13: Comparison of the electrical properties of the neurons which exhibited APs in control (C37, C44), glutamate treated (G37, G44), serotonin treated (S37, S44), acetylcholine chloride treated (A37, A44), glutamate+serotonin treated (GS37, GS44), and glutamate+serotonin→ acetylcholine chloride treated (GSA44). Since the neurotransmitters were added on day 30 after the cells were plated, the numbers 37 and 44 indicate 7 and 14	

days respectively after culturing the cells in the presence of neurotransmitters. The values are expressed as Mean \pm SE.....364

CHAPTER 1: GENERAL INTRODUCTION

Tissue engineering is a complex interdisciplinary field[1]. According to the definition given by Robert Langer and Charles Vacanti in their review paper in the May 14, 1993 issue of *Science*, “Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”[2, 3]. Before this definition was coined, at the 1992 UCLA symposium on tissue engineering, “Eugene Bell defined tissue engineering in terms of a more specific list of goals”[4, 5]:

1. “Providing cellular prostheses or replacement parts for the human body”.
2. “Providing formed non-cellular replacement parts capable of inducing regeneration”.
3. “Providing tissue or organ-like model systems populated with cells for basic research and for many applied uses such as the study of diseased states using aberrant cells”.
4. “Providing vehicles for delivering engineered cells to the organism”.
5. “Surfacing non-biological devices to create hybrid devices and systems”[4, 5].

Tissue engineering has an interdisciplinary root. Tissue engineering has evolved from the integration of knowledge from diverse fields namely; cell and developmental biology, basic medical and veterinary sciences, transplantation science, biomaterials, biophysics, biomechanics and biomedical engineering[6]. Figure 1 indicates how these diverse areas of science, engineering and

clinical medicine, contribute to the development of tissue engineering.

Success of tissue engineering is dependent upon successful cell culture technologies, biocompatible substrates and interfacing biological components with bio-hybrid systems [2, 3, 6].

Since tissue engineering involves constructing tissue and/or organ-like model systems as well as defining the surface of non-biological devices to create hybrid devices and systems, the major prerequisites to successfully achieve these goals are the following:

1. Developing a uniform cell isolation and cell culture methodology.
2. Understanding the growth factor requirements of the cells and developing defined mediums.
3. Understanding cell-cell and cell-matrix interactions to develop advanced biomaterials.
4. Employing surface engineering tools to characterize and modify the surface properties of biomaterials.
5. Enabling micro-fabrication technologies and interface design for integrating live cells with bio-hybrid (bio-MEMS) devices for advance research in tissue engineering, development of biosensor devices and designing the next generation of prosthetic devices.

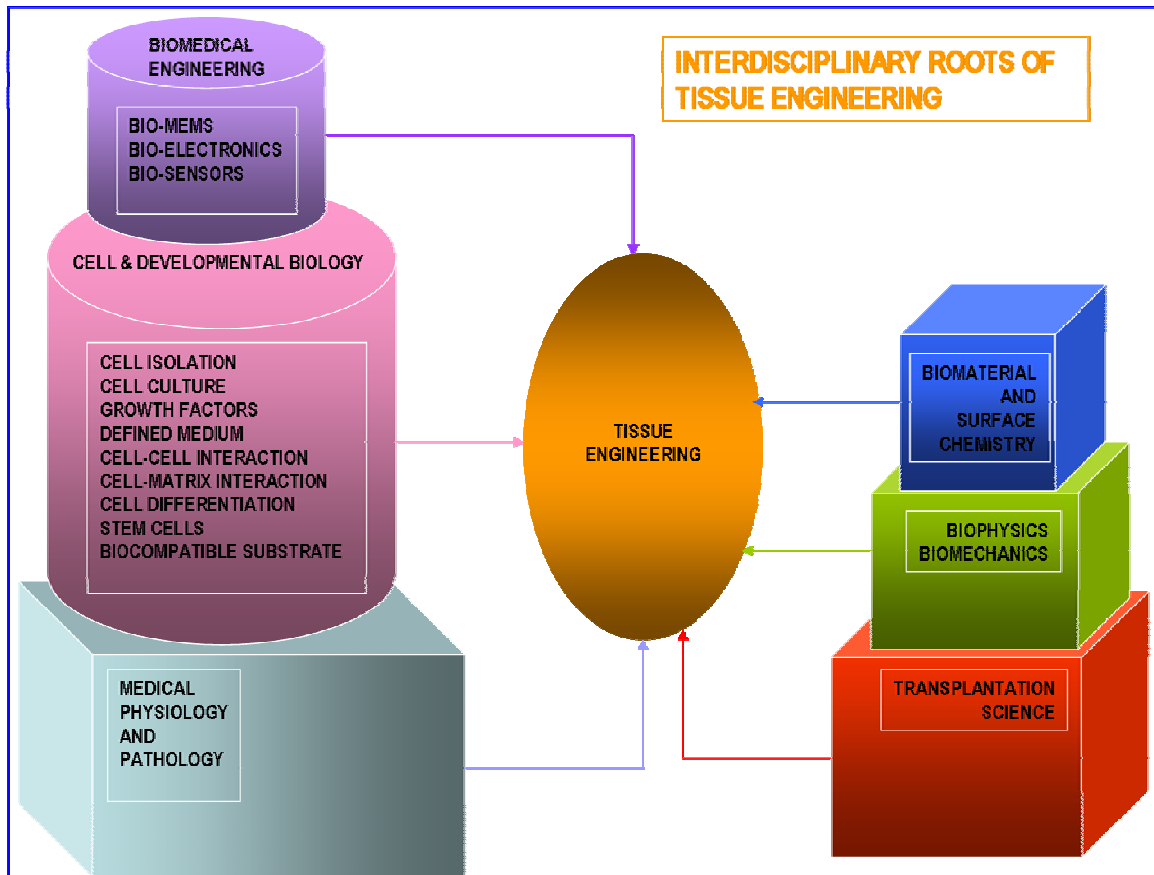


Figure 1. Interdisciplinary roots of tissue engineering

During last 15 years, efforts have been made by different research groups to develop *in vitro* micro-scale tissue engineered systems that combines the use of a defined medium with a chemically defined surface[7-25]. The main objective for developing such defined tissue engineered systems is to study cell differentiation, cell signaling, physiological information processing and to fabricate biological networks and bio-hybrid devices by patterning cells in a desired geometry. Most of the efforts have been made to develop such systems with a single cell type such as brain neurons, sensory neurons, retinal neurons, endothelial cells and cardiomyocytes[7-24, 26-51].

The next level of challenge in micro-scale tissue engineering is to develop *in vitro* systems to study the interaction of two, three or more cell types in a defined system that will more closely approximate an *in vivo* tissue of biological subsystem. Some simple examples are attempts to study the interaction between brain neurons and spinal cord neurons, motoneuron and sensory neurons, bipolar neurons and the amacrine cells of the eye, sympathetic neurons with heart muscle, renal sympathetic neurons and the renal vessels, the tubules, and the juxtaglomerular granular cells of kidney. It is feasible to study such cellular interactions by fabricating patterned networks of interacting cell types on a bio-hybrid device. The objective for building these tissue engineered networks is to mimic the different interacting organ systems of a body. Such defined tissue engineered systems could be used as powerful tools in getting a better understanding of the complex physiology that exists in mammalian systems. In this dissertation research work, an

attempt has been made to fabricate a defined, micro-scale tissue engineered system to study the interaction of two different interacting cell types.

The specific theme of this dissertation research was to tissue engineer the motoneuron to muscle segment of the stretch reflex arc circuit utilizing micro-fabrication, interface design and defined medium formulations. In order to address this central theme, the following hypothesis has been proposed. The first part of the hypothesis is that microfabrication technology, interface design and defined medium formulations can be effectively combined to tissue engineer the motoneuron to muscle segment of the stretch reflex arc. The second part of the hypothesis is that different growth factors, hormones, nanoparticles, neurotransmitters and synthetic substrate can be optimally utilized to regenerate the adult mammalian spinal cord neurons so as to replace the embryonic motoneurons in the stretch reflex tissue engineered construct with adult motoneurons.

The concept of the reflex circuit was originated in 16th century with Descartes[52], who believed that animals were mechanical devices that transformed sensory stimuli into motor responses. In 1924, British physiologist Charles Sherrington and his student E.G.T Liddell described the stretch reflex circuit[53-55]. The stretch reflex circuit is one of the most primitive circuits of mammalian system and serves mainly to control the length of the muscle. It consists of four elements: the stretch sensor (muscle spindle/ intrafusal fiber lie parallel between extrafusal, contractile musculature), extrafusal muscle fiber, sensory neuron and motoneuron (see figure 2). The basic

principle of the stretch reflex arc circuit is as follows: whenever there is a sudden stretch in a muscle, it needs to compensate back to its original length so as to prevent any kind of injury. It performs this compensation process using a simple negative feed back circuit called the stretch reflex arc. Any form of stretch in a muscle activates the stretch sensors (muscle spindle/ intrafusal fiber) lying deep in each muscle. After the stretch sensors get activated, it sends a train of signals to the spinal cord through the sensory neurons. The sensory neurons relay this information to the motoneuron.

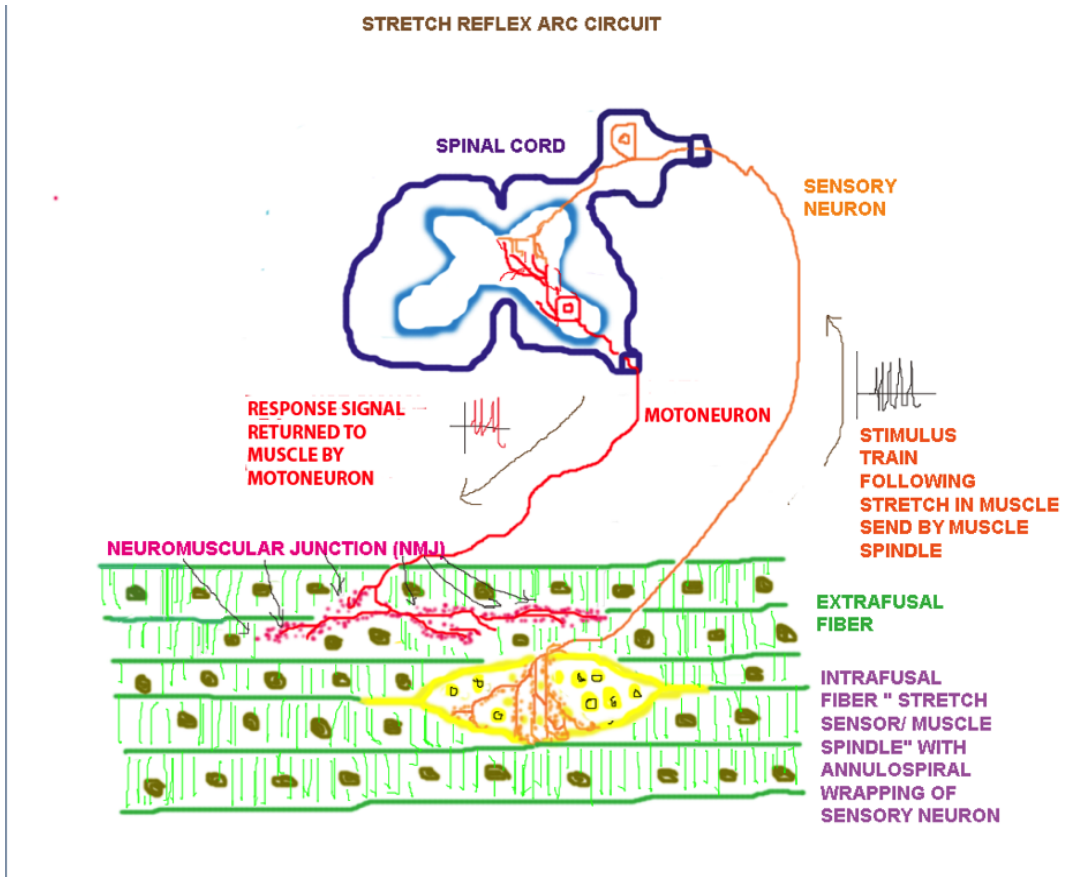


Figure 2. Stretch reflex arc circuit

The motoneuron performs the necessary information processing and sends the message to the extrafusal fibers so as to compensate for the sudden stretch action. The motoneuron conveys this message to the extrafusal fibers by communicating through the special synaptic junctions called neuromuscular junctions. Based on this information, the extrafusal fibers act accordingly so as to counter the effect of sudden stretch. This is also called the monosynaptic stretch reflex that involves a single synapse between a sensory neuron and a motoneuron. The reflex time of the monosynaptic reflex is extremely short (about 20 ms)[56, 57]. Formation of most of the stretch reflex circuits in animals are developmentally regulated by different growth factors and cell types in a temporal fashion. Hence to date studying these stretch reflex circuits is only feasible in animal models[58-74]. Although recent advancements in developmental biology has shed new lights on the development of such circuits, almost no effort has been made to tissue engineer such circuits for a better understanding of the complex development and repair processes of the stretch reflex circuit formation[75].

The long-term goal of this research is to tissue engineer a cellular prototype of the entire stretch reflex circuit. However achieving this goal was not possible in a single dissertation, so the focus of my dissertation research was on two main objectives. The first goal was to develop a cantilever-based bio-MEMS device to measure communication between a motoneuron and myotube in a defined medium formulation (supplemented with specific growth factors) (Figure 3). In figure 3, the experimental system design has been shown. Such a circuit would be able to act as a defined *in*

vitro tool in studying spasticity, spinal cord injury, amyotrophic lateral sclerosis (ALS) and for development of bio-mimetic prosthetics. The second goal was to develop tissue-engineering strategies using different growth factors, hormones, nanoparticles, neurotransmitters and synthetic substrate for growing adult mammalian spinal cord neurons, so that in the future, these adult motoneurons could replace the embryonic neurons in the stretch reflex arc circuit model. It is important to develop adult models so as to study the age related neurodegenerative diseases, neuro-muscular disorders and injuries.

The work has been divided into three independent parts which constitute the three main topics of the thesis. The three parts are as follows:

Part 1: Skeletal muscle tissue engineering

Part 2: Tissue engineering neuro-muscular junction (NMJ)

Part 3: Tissue engineering adult mammalian spinal cord

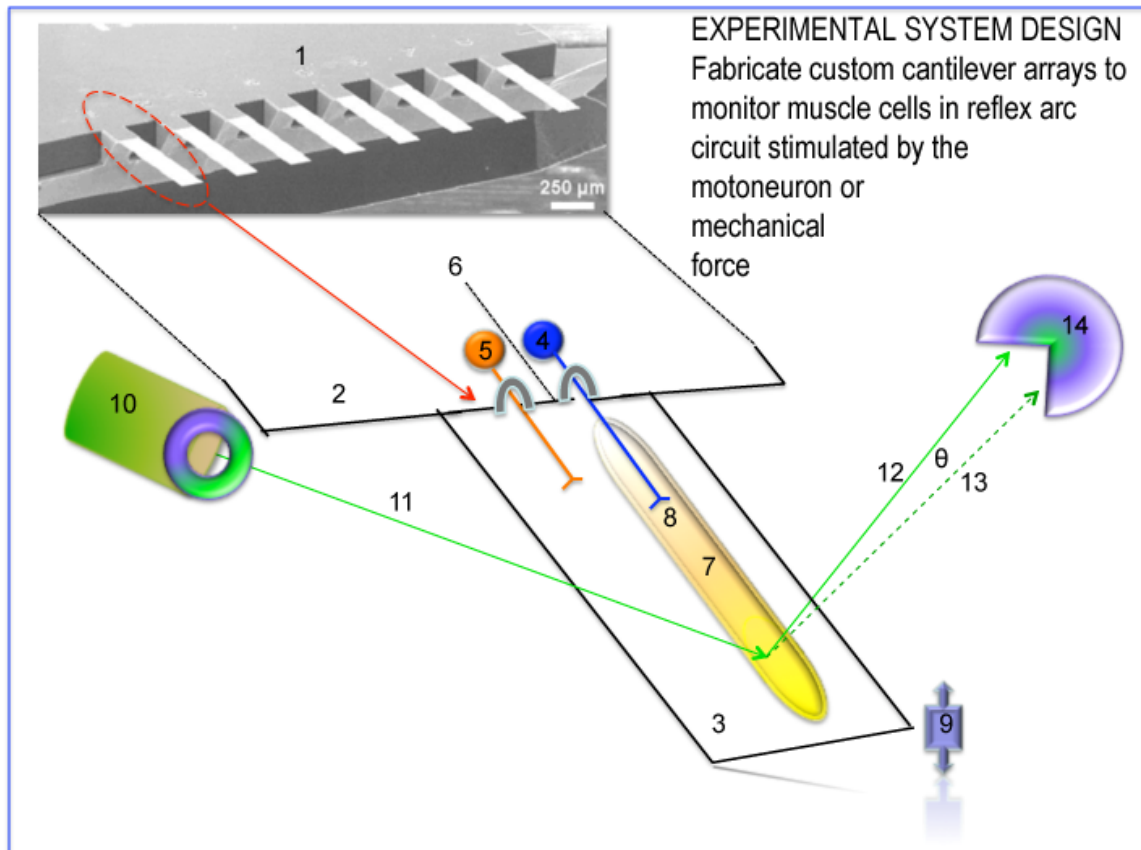


Figure 3. A cantilever-based bio-MEMS device to measure communication between a motoneuron and myotube. Each component of the system has been marked with a number (1-14). The detail of each part of the device has been explained below: 1. Custom cantilever arrays; 2. Detailed layout of pattern on cantilever array; 3. Individual cantilever; 4. Site for motoneuron plating on patterned substrate; 5. Site for sensory neuron plating on patterned substrate; 6. Barrier between motoneuron and sensory neuron plating sites; 7. Site for skeletal muscle myotube formation; 8. Putative neuromuscular junction site between motoneuron and skeletal muscle; 9. Deflection of cantilever due to muscle contraction after receiving signal from motoneuron; 10. Source for laser beam; 11. Laser beam falling on skeletal muscle on top of cantilever; 12. Reflecting laser beam; 13. Reflecting laser beam changes angle due to bending of cantilever caused by contracting myotubes; 14. Photo-detector measuring the change in angle θ

In part 1, skeletal muscle tissue engineering strategies have been discussed. This part consisted of four independent experiments (chapter 2-5). In this series of experiments, a chemically defined, serum-free medium was developed to grow functional myotubes from a dissociated culture of muscle cells. A synthetic substrate was used for this study. Myotubes were characterized morphologically, immunocytochemically and electrophysiologically. Further, myotubes were integrated with a cantilever based bio-hybrid device to study muscle physiology. In chapter 2, a systematic study was carried out describing the role of individual growth factors involved in myotube formation and the development of a defined medium for muscle culture. In chapter 3, the defined model system was utilized to study skeletal muscle differentiation[76]. In chapter 4, a detailed protocol for bio-hybrid device development and integration of the myotubes in the device has been documented[77]. In chapter 5, a significant improvement had been made in medium formulation that has enabled long term survival of myotubes, structural development of the excitation-contraction (E-C) coupling apparatus as well as neonatal myosin heavy chain (MHC) expression. This system could be used as a powerful tool to study skeletal muscle differentiation, skeletal muscle tissue engineering, muscular dystrophy and to carry out functional assay of the myotubes.

In part 2, mammalian neuromuscular junction (NMJ) tissue engineering strategies have been developed. In these experiments, for the first time, a defined medium had been developed which promoted in *vitro* mammalian NMJ formation. It has been achieved by systematic integration of the

media developed in part 1 with additional factors known to promote neuronal growth. Further, a simple, novel culture technique had been documented to coculture motoneuron and muscle. This part consisted of two independent experiments (chapters 6-7). In chapter 6, the first preliminary medium was developed which promoted neuromuscular junction formation[78]. In chapter 7, a significant improvement was made over the preliminary medium. The improved medium detailed in chapter 7 promoted neonatal myosin heavy chain expression in muscle, longer survival and increased neuromuscular junction formation. These are the first chemically defined medium which promoted mammalian neuromuscular junction formation *in vitro*. Further, this is the first evidence which shows that neuromuscular junction formation can be achieved on a synthetic substrate which is free from all known extracellular matrix components. These studies offers a basic system that can be utilized for studying the communication between the motoneuron to muscle segment of the stretch reflex circuit, nerve-muscle tissue engineering, regenerative medicine and development of limb prosthetics.

In part 3, tissue engineering strategies have been developed for *adult* mammalian spinal cord cells. In this set of experiments, functional adult mammalian spinal cord neurons have been grown using a synthetic silane substrate and a medium was developed using different growth factors, hormones, nanoparticles and neurotransmitters. This part consisted of three experiments (chapter 8-10). In chapter 8, a chemically defined, serum-free medium was developed to culture adult rat spinal cord neurons on a synthetic, silane substrate. The cells were characterized morphologically,

immunocytochemically and electrophysiologically. Thirty percent of the neurons were electrically active in this culture model[79]. In chapter 9, the neuroprotective activity of Cerium oxide nanoparticles in adult rat spinal cord neurons in culture was assessed. The culture system developed in chapter 8 was used for this experiment. A significant increase in the neuronal survival was observed following nanoparticle treatment. This finding raised the possibility that in the future, Cerium oxide nanoparticles could be used in nanomedicine for spinal cord therapy[80]. In chapter 8, only 30% of the neurons were electrically active. This would limit the use of this model for functional studies. In chapter 10, the role of neurotransmitters in enhancing the functionality of the adult mammalian spinal cord neurons in culture was discovered. It was shown that the electrical activity of most of the neurons could be restored by applying multiple neurotransmitters in a temporal manner. Sixty percent of the neurons regained their electrical activity by temporal application of multiple neurotransmitters. For the first time; it was shown that exogenous application of neurotransmitters could be used as a therapeutic tool in tissue engineering nerve constructs and in regenerative medicine. Apart from documenting the work in “Experimental Neurology”[81], a patent has been filed on this work.

In chapter 11, these advance muscle and nerve tissue engineering technologies are discussed in context of translational research as useful biomedical engineering tools in studying the stretch reflex arc circuit, neuromuscular junction formation and function, spinal cord injury, muscle development, muscle disorders and in developing advance strategies in regenerative medicine.

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Part 1: Skeletal Muscle Tissue Engineering

CHAPTER 2: DEVELOPMENT OF A NOVEL SERUM-FREE CELL CULTURE MODEL OF SKELETAL MUSCLE DIFFERENTIATION BY SYSTEMATIC INVESTIGATION OF THE ROLE OF DIFFERENT GROWTH FACTORS IN MYOTUBE FORMATION

Introduction

The major goal of this study is to develop a serum-free cell culture model to study skeletal muscle differentiation and to get a better understanding of the process by which individual skeletal muscle cells migrate and align together to form functional, contractile, multinucleated myotubes [1-11]. The serum-free defined cell culture model involved the development of a chemically defined medium supplemented with different growth factors and the use of a synthetic, biocompatible silane substrate for cell growth. We have formulated the chemically defined medium by systematically studying the effects of individual growth factors on myotube formation in vitro. The biocompatible silane substrate, N-1[3-(trimethoxysilyl) propyl] diethylenetriamine (DETA) was developed to provide a uniform growth surface for mammalian cell types as diverse as hippocampal neurons and endothelial cells [12, 13]. Furthermore, the ease of applying photolithographic patterning techniques to these biomimetic substrates facilitates controlled cellular adhesion and outgrowth [13-17].

In vivo and in vitro studies carried out in last two decades have indicated that skeletal muscle differentiation involves the specific interaction of multiple growth factors with both the myocyte and the subsequently developing myotubes. The different growth factors which have been implicated in

skeletal muscle differentiation includes vitronectin, bFGF, CT1, GDNF, BDNF, NT3, and NT4 [1-10, 18]. Specifically, the role bFGF plays in limb morphogenesis has been elucidated [19, 20]. Additionally, the roles of NT3 and NT4 in the survival and normal development of skeletal muscle fibers has been documented [21, 22].

Although skeletal muscle development is a very well studied model, limited success had been achieved in generating different muscle phenotypes in culture [7, 8]. This limited success was mostly due to non-availability of a defined culture model. All the culture models relied heavily on the use of serum [23-41]. The use of serum further posed a serious challenge in successful tissue engineering of the skeletal muscle for therapeutic applications. Till date, no systematic in vitro study has been carried out optimally combining different growth factors to develop a chemically defined medium. In this study we have analyzed the effects of bFGF, CT1, GDNF, BDNF, NT3, NT4, and vitronectin on myocyte fusion and skeletal muscle differentiation.

Through this analysis, we developed a simple chemical and growth factor based medium formulation and proposed a novel technique which promotes formation of robust, functional, contractile, multinucleated myotubes in culture. We have immunocytochemically characterized the different morphologies of myotubes using embryonic myosin heavy chain (MHC) antibody (F1.652). We further observed that this novel technique, which promoted robust myotube formation, also supported nerve-muscle coculture. We had provided immunocytochemical evidences of muscle-

nerve coculture. We believe this chemically defined formulation and the proposed development model will be useful tools in studying myocyte biocompatibility, muscle differentiation, myopathies, muscle tissue engineering and neuromuscular junction formation.

Methods and Materials

Surface modification

Glass coverslips (Thomas Scientific 6661F52, 22 x 22mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried[42, 43].

Surface characterization

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1s and C1s scans, utilizing monochromatic Al K α excitation, were obtained.

Skeletal muscle culture and serum free medium

The skeletal muscle was dissected from the thighs of the hind limbs of fetal rat (17-18 days old). The tissue was collected in a sterile 15-ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated

using 1 ml of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath (100 rpm). After 30 minutes the trypsin solution was removed and 2 ml of L15 + 10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated. The supernatant was then transferred to a 15-ml centrifuge tube. The same process was repeated two times by adding 2 ml of L15 + 10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300 g for 10 minutes at 4°C. The pellet obtained was washed 5 times with L15 medium then resuspended in 10 ml of L15 and plated in 100 mm uncoated dishes for 30 min. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and plated on the coverslips. The cells were plated at a density of 700-1000 cells/mm². The cells attached to the substrate in 1 h. The serum-free medium (containing different growth factors) was added to the culture dish after 1 h and the cells were maintained in a 5% CO₂ incubator (relative humidity 85%). Half of the medium was change after every 4 days[43].

Coculture of three cell types in the defined medium

We developed a simple coculture technique for the growth and differentiation of skeletal muscle* DRG(Sensory Neurons)* Spinal Cord. We mix the dissociated muscle cells with dissociated sensory neurons (DRG) and the spinal cord cells. The sensory neurons (DRG) isolation protocol was described in earlier paper[44]. In brief, DRG was isolated from embryonic day 14 (E14) rat embryos and dissociated using trypsin and the resulting single cell suspension was mixed with the

dissociated muscle cells in the serum-free medium. Similarly, we dissected out the whole spinal cord from E14 embryo and dissociated it in trypsin solution, and subsequently the single cell suspension of spinal cord cells were mixed with the mixture of dissociated muscle and DRG cell suspension. The combined three cell suspension was then plated in the coverslips at a density of 800 cells/mm². After 30 minutes, the well containing coverslips were filled with serum-free medium (Figure 4). The first medium change was done at day 4 as described in figure 4 and the cultures were maintained for 3 weeks.

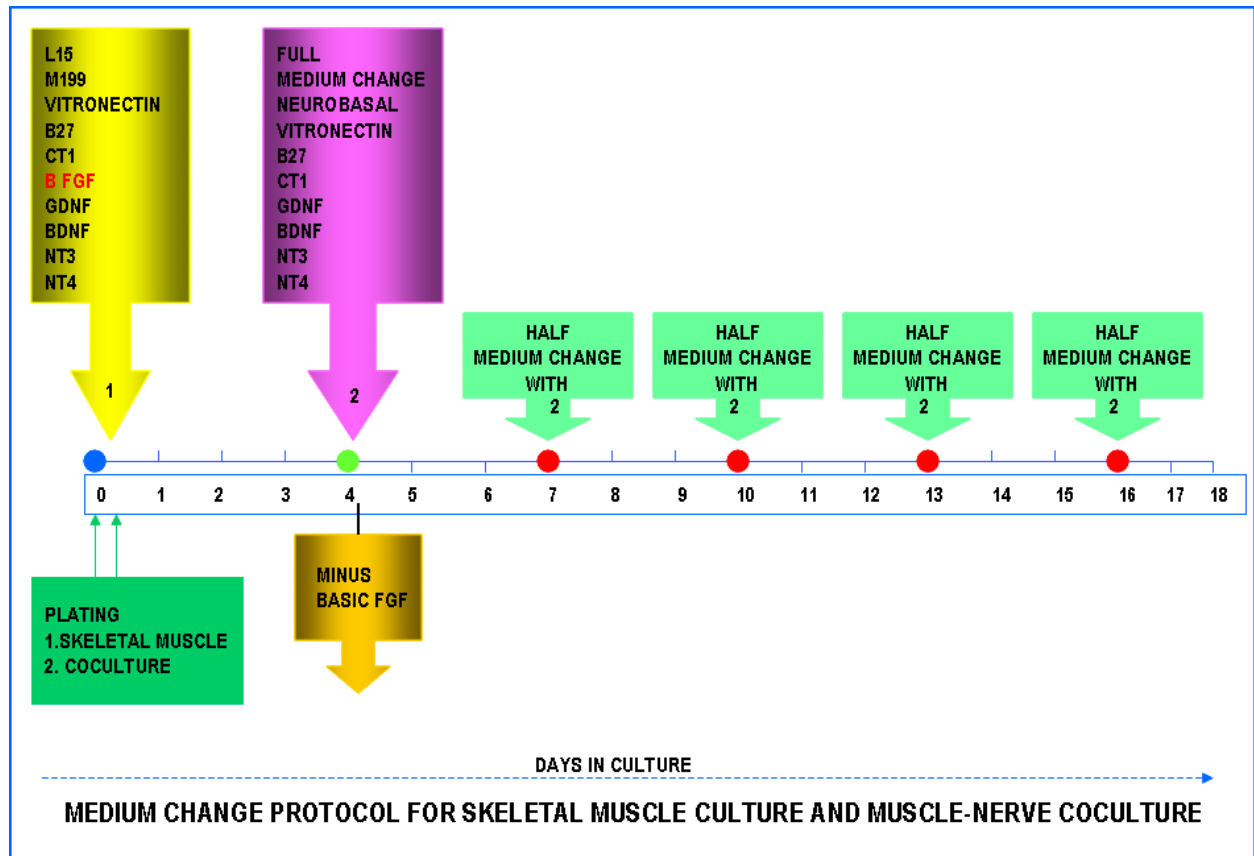


Figure 4. Flow chart showing the technique to grow robust myotubes and muscle-nerve cocultures: The formulation IX and the medium change protocol which is most optimal for myotube and coculture growth.

Immunocytochemistry of skeletal muscle

Coverslips were prepared for immunocytochemical analysis as previously described[43]. Briefly, coverslips were rinsed with PBS, fixed in -20°C methanol for 5-7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 10 minutes, and blocked for 2h with 10% goat serum and 1% BSA. Cells were incubated overnight with primary antibodies against embryonic myosin heavy chain (F1.652) (Developmental Studies Hybridoma Bank) diluted (1:5) in the blocking solution. Cells were washed with PBS and incubated with the secondary antibody (Cy3 conjugated anti-mouse, Jackson Labs., 1:200 dilution in PBS) for 2 hours. After 2 hours the coverslips were rinsed with PBS and mounted on glass slides and observed in confocal microscope.

Immunocytochemistry of cocultures

Double staining with neurofilament 150 and embryonic myosin heavy chain: Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4 °C with rabbit anti-neurofilament M polyclonal antibody, 150 kD (AB1981, diluted 1:2000; Millipore/Chemicon, Temecula, CA, USA) and fetal MHC (F1.652, IgG, Developmental Studies Hybridoma Bank, diluted 1:5). After overnight incubation, the coverslips were rinsed three times with PBS and then incubated with the appropriate secondary antibodies for 2 h. After rinsing three times in PBS, the coverslips were mounted with Vectashield_DAPI mounting medium onto glass

slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative.

AChR labeling of the myotubes

AChRs were labeled as described previously[14] by incubating cultures with 5×10^{-8} M of α -bungarotoxin, Alexa Fluor® 488 conjugate (B-13422; Invitrogen/Molecular Probes, Carlsbad, CA, USA) for 1.5 h at 37 °C before observation. Following incubation in α -bungarotoxin, the cultures were fixed as mentioned above for further staining with embryonic myosin heavy chain (F1.652) antibodies.

Results and Discussion

DETA surface modification and characterization

Static contact angle and XPS analysis was used for the validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles ($40.64^\circ \pm 2.9$ /mean \pm SD) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results[42, 43, 45-47]. Based on the ratio of the N (401 and 399 eV) and the Si $2p_{3/2}$ peaks, XPS measurements indicated that a monolayer of DETA was formed on the coverslips.

Development of serum free medium formulation

The results have been summarized in Table 1. In the following paragraph we will discuss the effects of different growth factors present in nine different formulations. Unless otherwise stated, half of the medium was changed every three to four days.

Formulation I (F I) was the basal medium. It consisted of Leibovitz's medium and M199 in 3:1 ratio (v/v). The basal medium did not promote myotube formation. The cells died after 4 days in culture.

In formulation II (F II) vitronectin was added to the basal medium. Myotube formation was not observed. Cells did not survive in the culture for more than 4 days.

In formulation III (F III) B27 supplement was added to formulation II (F II). The cells survive for 8-10 days but there was no myotube formation.

Table 1. Development of chemically defined serum-free medium by systematically adding individual growth factors in the culture. Each of these experimental trials had been carried out atleast 6 times ($n \geq 6$).

F	Components of the Medium Formulation									Myotube Properties
	L15:M199 3:1	Vt	B27	bFGF	CT1	BDNF	GDNF	NT3	NT4	
I	+	*	*	*	*	*	*	*	*	(---)
II	+	+	*	*	*	*	*	*	*	(---)
III	+	+	+	*	*	*	*	*	*	(- +)
IV	+	+	+	+	*	*	*	*	*	(+++)
V	+	+	+	*	+	*	*	*	*	(+)
VI	+	+	+	+	+	*	*	*	*	(±±±)
VII	+	+	+	+	+	+	+	*	*	(±±±±)
VIII	+	+	+	+	+	+	+	+	+	(±±±±)
IX	+	+	+	+	+	+	+	+	+	(±±±±±)

The following abbreviations and signs had been used in the table.

F: Formulation number indicating the components added in the medium. If it indicates “+” sign, that means the factor had been added. If it indicates “*”, that means the component had been omitted.

L15: Leibovitz’s medium

Vt: Vitronectin

B27: B27 supplement

bFGF: Basic fibroblast growth factor

CT1: Cardiotrophin 1

BDNF: Brain derived neurotrophic factor

GDNF: Glial derived neurotrophic factor

NT3: Neurotrophin 3

NT4: Neurotrophin 4

(---): No myotube formation

(- +): No myotube formation but cells survive for 8-10 days

(+): Small myotubes consisting of 4-6 nuclei

(+++): Robust myotube formation (10-30 nuclei) but along with that lot of fibroblast also proliferated in the culture

(±±±): Robust myotube formation (10-30 nuclei) with reduction in fibroblast proliferation

(±±±±): Robust myotube formation (10-30 nuclei) and significant reduction in fibroblast proliferation

(±±±±±): Robust myotube formation (10-30 nuclei), minimal fibroblast proliferation and further supports nerve-muscle co-culture

In formulation IV (F IV) bFGF was added to formulation III (F III). Addition of bFGF led to formation of robust myotubes. Myotubes started appearing by day 2 in culture. Contracting myotubes were observed by day 4. Fifty percent of the total area of the coverslip was covered with the myotubes. The myotubes survived for 10-12 days in culture. Many myotubes popped out off of the coverslip due to extensive contractions. Extensive proliferation of fibroblasts was also observed in the culture.

In formulation V (F V) CT1 was added to formulation III (F III). Addition of CT1 lead to formation of small myotubes with 4-6 nuclei. Myotubes started appearing by day 2 in culture. By day 4 most of these small myotubes exhibited mild contractions. Ten percent of the total area of the coverslips had contractile myotubes. Myotubes survived for 6-7 days in culture. Formulation V had no bFGF in it, but we observed small myotube formation even without its presence.

In formulation VI (F VI) both bFGF and CT1 were added to the formulation III (F III). Robust myotube formation was observed with reduction in fibroblast proliferation. Fibroblast proliferation was less as compared to formulation IV (F IV) where only bFGF was added. Sixty percent of the total surface area of the coverslips were covered with contractile myotubes. Most of the myotubes started contracting by day 4. Myotubes exhibited extensive contractions and survived for 10-12 days in the culture.

In formulation VII (F VII) GDNF and BDNF were added to formulation VI (F VI). Robust myotube formation and significant reduction in fibroblast proliferation was observed. The presence of GDNF and BDNF significantly reduced fibroblast proliferation and increased the total surface area covered with myotubes. Almost 65% of the total surface area of the coverslip was covered with contractile myotubes and most of the myotubes initiated contractions by day 4.

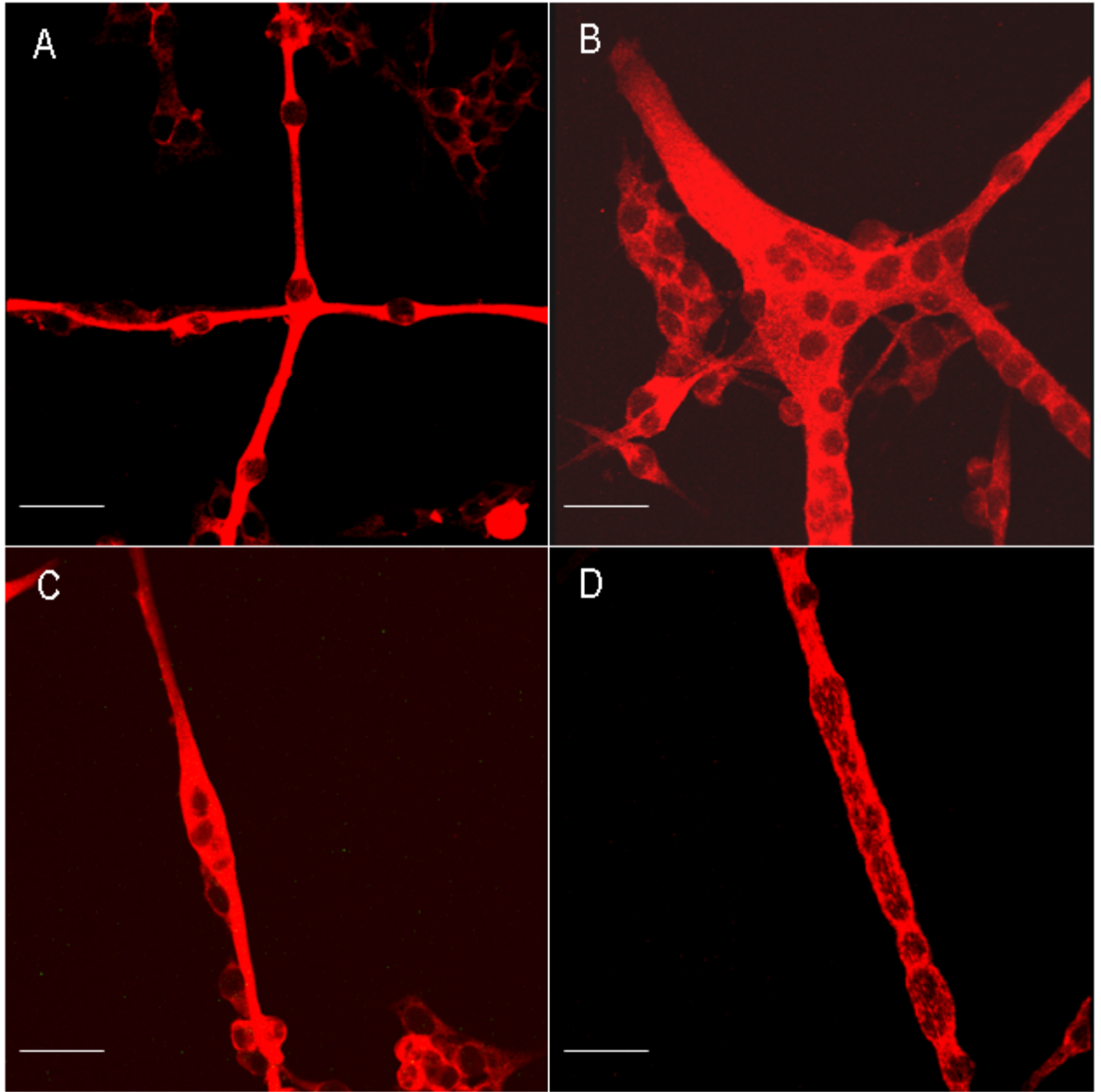
In formulation VIII (F VIII) NT3 and NT4 were added to formulation VII (F VII). We did not observe any significant qualitative difference from formulation VII (F VII). Functionally, the myotubes started contracting by day 2. Additionally, significantly reduced fibroblast proliferation was observed compared to formulation III (F III).

In formulation IX (F IX) no additional growth factors were added; instead two changes were made in growth factor application. First, instead of replacing half the medium during the first change, the entire medium was replaced. Second, in the process of changing the whole medium, bFGF was withdrawal, resulting in the following formulation: Neurobasal/ vitronectin/ B27/ CT1/ GDNF/ BDNF/ NT3/ NT4. These two changes brought about a significant increase in the total number of myotubes. The final medium formulation has been enumerated in Table 2 and the medium change protocol has been described in the flowchart indicated in Figure 4. By day 6, 90% of the total surface area of the coverslip was covered with robust, contractile myotubes. Additionally, there was minimal fibroblast contamination. The contractions started by day 2. The myotubes survived in the

culture for 16-20 days. All of the different morphologies of myotubes observed in the culture were stained with embryonic myosin heavy chain antibodies (Figure 5 A-D). Chain-like (Figure 2.2 A), branched (Figure 5 B), spindle shaped (Figure 5 C) and cylinder like (Figure 5 D) morphologies of myotubes were observed in the culture. The clustering of acetylcholine receptors on the membrane surface of the different morphologies of myotubes are shown in figure 4 E-H. All the myotubes showed clustering of acetylcholine receptors on their membrane surface.

Table 2: Composition of novel serum-free medium for a 500 ml sample.

S. No.	Component(s)	Catalogue #	Company	Amount
1.	L15	Invitrogen	11415064	375 ml
2.	M199	Invitrogen	11150059	125 ml
3.	Vitronectin	Sigma	V0132	50 µg
4.	B27	Invitrogen	17504044	10 ml
5.	Basic FGF	Invitrogen	13256029	10ng/ml
6.	CT1	Cell Sciences	CRC700B	10 µg
7.	GDNF	Invitrogen	10907012	10 µg
8.	BDNF	Invitrogen	10908019	10 µg
9.	NT3	Cell Science	CRN 500B	10 µg
10.	NT4	Cell Science	CRN 501B	10 µg
11.	Sodium Bicarbonate	Fisher	5233500	0.7 g
12.	Osmolarity			320-325 mOsm
13.	pH			7.3



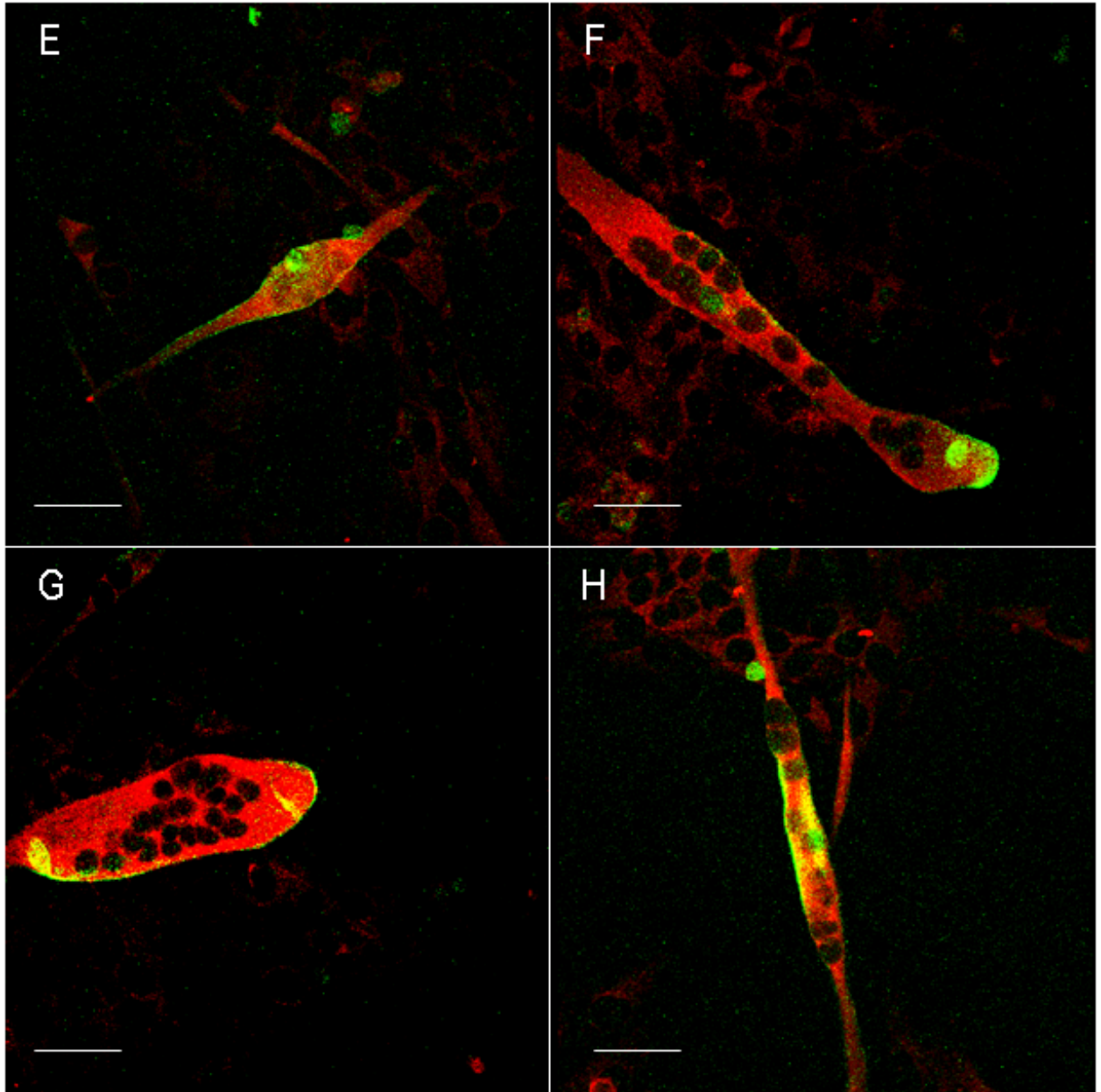


Figure 5. Different morphologies of myotubes stained with embryonic myosin heavy chain antibodies (Red) and clustering of acetylcholine receptor (Green) on the membrane surface of myotubes. Scale bar was 50 μm . A. Chain like morphology of myotubes. B. Branched morphology of myotubes. C. Spindle shaped morphology of a single myotube. D. Cylinder shaped morphology of a single myotube. E-H. Different morphologies of myotubes showing the clustering of acetylcholine receptors (Green) on the membrane surface of different myotubes.

Medium formulation IX (F IX) and the subsequent change at day 4, further supported nerve-muscle co-culture (Figure 6 A-F). In figure 6 A, we observed the sensory neurons along with the myotubes in the cultures. The sensory neurons were seen as large cell bodies. In figure 6 B, the neuron process is shown to run parallel to the myotubes. In figure 6 C, multipolar cell morphologies of motoneurons, along with the large sensory neurons were observed. In figure 6 D, branched, striated myotubes, and the neuron process crossing over the myotube is shown. In figure 6 E-F wrapping of neuron process on the three dimensional structure of myotubes indicated the possibility of their innervation.

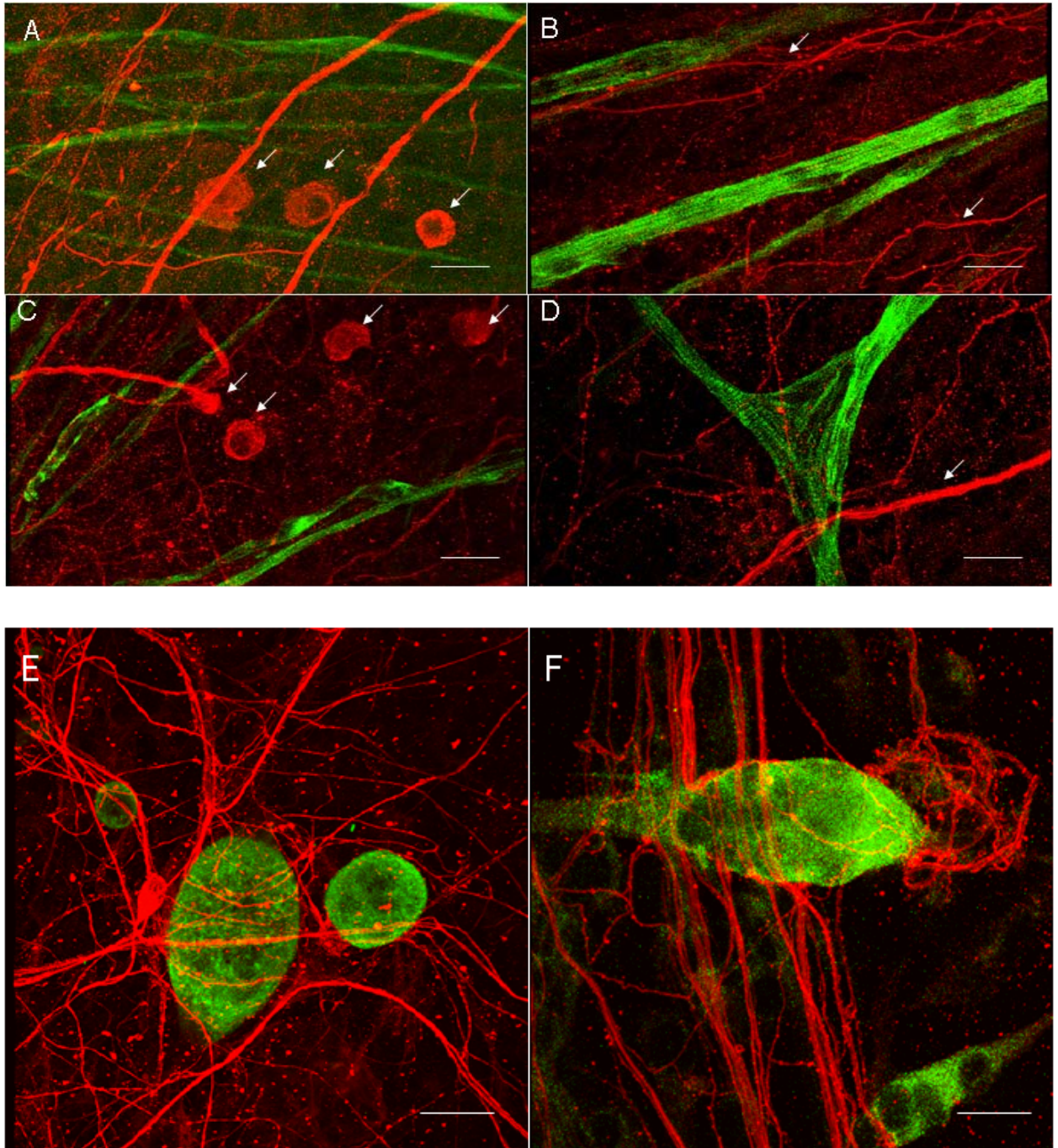


Figure 6. Coculture of skeletal muscle* sensory neurons (DRG)* spinal cord neurons: All the neurons were stained with antibody against neurofilament-M (150 KD) (Red) and the myotubes were stained with antibody against embryonic myosin heavy chain (F 1.652) (Green). Scale bar for was 75 μm . A. The large sensory neurons (DRG) were seen stained with NF 150 (Red). The neurons are shown with white arrow. The myotubes stained with F1.652 (Green) were seen in the same field. B. The neuron process in red were seen running parallel with the myotubes (Green).C. The multipolar motoneuron was seen in close proximity with the myotubes. In the frame we also observed multiple large sensory neurons. All the neurons were pointed out by white arrows. D. A bundle of multiple processes of neurons crossed the striated, branched myotube. E. A single multipolar motoneuron was observed to form a wrapping around the myotube.F. Neuron processes formed wrapping on the myotubes.

In this study we have described the step-by-step development of a novel serum-free in vitro cell culture system resulting in the formation of robust, contracting, multinucleate myotubes from dissociated skeletal muscle cells obtained from hind limbs of fetal rats. Step-by step development consisted of experimentally evaluating the effect of individual growth factors on myocyte survival and subsequent myotube formation. We concluded the study by showing that the most optimal formulation i.e formulation IX (F IX) supported muscle as well as nerve-muscle co-culture growth.

We have empirically developed a serum-free medium supporting the survival, proliferation and fusion of fetal rat myoblasts into contractile myotubes. The rational for selecting these growth factors (vitronectin, B27, bFGF, CT1, GDNF, BDNF, NT3 and NT4) is solely based on the distribution of their receptors in the developing myotubes in rat fetus.

Role of Vitronectin

We added vitronectin to the culture medium because vitronectin receptors promotes cell adhesion and fulfill an anchoring function during the skeletal muscle differentiation process in vitro[48, 49]. Addition of vitronectin by itself did not promote myotube formation.

Role of B27

Previously we had documented that B27 supplement supported cardiomyocyte growth[46]. So B27 supplement was added in the culture medium as a serum-replacement. Addition of B27

supplement promoted cell survival but did not promote myotube formation.

Role of bFGF and the controversy concerning the role of bFGF in differentiation

Basic fibroblast growth factor (FGF-2) is a 17-kDa member of the heparin binding growth factors. Basic FGF plays a complex yet poorly understood role in skeletal muscle differentiation. Several studies have indicated that bFGF promotes limb development yet there have been *in vitro* studies indicating that bFGF promotes division of skeletal muscle cells but inhibits the differentiation process and it has been documented that terminal differentiation of the skeletal muscle occurs in G1 phase and is repressed by fibroblast growth factor [19, 20, 50-57]. Interestingly, in our study, even in the presence of bFGF, most myoblasts fused and differentiated to form functional myotubes. Our *in vitro* results support the hypothesis that there are at least two different pools of myoblasts present in the developing limb bud. In one population, bFGF promotes differentiation and in the other population it inhibits muscle differentiation.

Role of CT1

Cardiotrophin-1 (CT1) is a cytokine belonging to the IL-6 family. It is expressed at high levels in embryonic limb bud development and is secreted by differentiated myotubes. CT1 promotes cardiac myocyte survival, regeneration of extraocular muscle, exhibits increased immunoreactivity in regenerating muscle and promotes motoneuron survival [58-66]. In this study we observed that addition of CT1 to basal medium/ vitronectin/ B27 promoted formation of small myotubes. It

indicates that CT1 by itself has the potential to initiate myotube formation but supports from other growth factors is required.

Role of GDNF and BDNF

Glial cell line derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-beta superfamily. GDNF plays a role in the differentiation and survival of central and peripheral neurons and in kidney organogenesis. GDNF is widely expressed in developing skeletal muscle and is involved in regulating the distribution of acetylcholine receptors in mouse primary skeletal muscle cells[67-71].

Brain-derived neurotrophic factor (BDNF) is a ligand for the low-affinity NGF receptor, p75, and for the high affinity neurotrophin receptor, trkB. It is expressed in developing skeletal muscle, promotes motoneuron survival and also plays a vital role in the formation of the neuromuscular junction. BDNF rescues myosin heavy chain IIB muscle fibers after neonatal nerve injury [72-74] and along with NT4 promotes phenotypic recovery of fast and slow twitch fibers. We believe that the above mentioned effects of GDNF and BDNF promotes enhanced myotube formation in the culture.

Role of NT3 and NT4

Neurotrophin-3, or NT-3 is a neurotrophic factor, in the NGF (Nerve Growth Factor)-family of neurotrophins. It is one of five neurotrophin growth factors which shape the development of the

nervous system by regulating neuronal survival and differentiation [22]. Recent studies have indicated that NT-3 has essential non-neuronal function. It plays a key role in cardiac development[75]. NT4[21, 72, 73] promotes normal development of slow muscle fiber phenotypes and phenotypic recovery of fast and slow twitch fibers.

Our studies have indicated that addition of NT3 and NT4 results in early contractions of the myotubes. We are speculating that NT3 and NT4 have a synergistic effect in muscle differentiation.

Co-culture studies

One interesting feature of the differential application of growth factors in formulation IX (F IX) is that it supported the survival and growth of skeletal muscle, sensory neurons and spinal cord motoneurons in a three cell co-culture model. While this seems intuitive, it is a crucial finding for easily building functional muscle-motoneuron, muscle-sensory neuron and muscle-motoneuron-sensory neuron constructs and for ultimately reconstructing the stretch reflex arc *in vitro*. Previous implementation of the multi-cell type co-culture experiments utilized serum containing mediums and followed complex culture techniques[76-81]. This defined medium offers a uniform medium formulation and a simple technique to study the interaction between multiple cell types. Further the use of defined medium offers a test bed for studying and quantifying the cell-cell interaction in a much more controlled environment in the absence of serum and in the presence of a synthetic growth substrate. The use of synthetic growth substrate offers a method to quantify the role of extracellular matrix structure in forming synapses between nerve and muscle. The use of synthetic

substrate offers a simple technique to integrate the culture system with bio-MEMS devices for hybrid system development. Our current efforts are to refine this medium so as to create the motoneuron-muscle connection, muscle to sensory neuron connections and to study neuromuscular junction in a defined system. Current three cell type coculture model includes the glial cells in the culture. In future studies, we will refine the culture by studying the effect of glial cells neuromuscular rjunction formation, muscle differentiation and muscle spindle formation.

This work documents the development of a medium formulation resulting in robust myotube formation and provides an analysis of the role of the individual factors in that process. Furthermore, the myotubes develop a MHC profile which results in functional contractile properties. This is important developmentally, because it shows the system provides for physiological maturation of the myotubes and facilitates studies regarding the MHC class switching based on growth factor manipulation. Lastly, the final medium formulation was determined to support the growth of motoneurons and sensory neurons as well as their co-culture with myotubes. This finding is critical to developing high throughput bio-MEMS devices aimed at engineering and studying the stretch reflex arc *in vitro*. Consequently, this medium will be a powerful tool for nerve-muscle tissue engineering studies.

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CHAPTER 3: A DEFINED SYSTEM TO ALLOW SKELETAL MUSCLE DIFFERENTIATION AND
SUBSEQUENT INTEGRATION WITH SILICON MICROSTRUCTURES. BIOMATERIALS. 2006
AUG;27(24):4374-80.

Introduction

We are seeking to create hybrid biological/non-biological systems by integrating silicon devices with cellular components. In particular, we are interested in integrating components of the stretch reflex arc (skeletal muscle, muscle spindle, motoneuron and sensory neuron) with silicon-based devices for applications from spinal cord repair and prosthetics to biorobotics systems. To treat a cell as a component and to take advantage of a cell's plethora of capabilities requires understanding and manipulating its requirements in a controlled and reproducible fashion [1]. Cells can then be used as building blocks to create functional hybrid systems. In this report, we demonstrate the control of myocyte differentiation to create functional myotubes in a totally serum-free defined environment on a modified silicon dioxide substrate and on cantilever microstructures. This advance should allow the integration of this system with our previously published results regarding neuronal systems in order to begin investigating motoneuron to muscle actuation and their use in control systems [2-4]. Electrophysiological characterization indicates the myotubes have normal electrical activity. We believe by controlling the growth substrate and the composition of the medium the differentiation of the myocytes could be manipulated and one can now consider the idea of evolving functional hybrid materials from progenitor cells [5].

Primary culture of skeletal muscle has been a model system to study cell differentiation for many years [6-8]. Apart from its clinical relevance to myopathies and limb regeneration, primary muscle culture has recently received enormous attention from multi-disciplinary fields such as tissue engineering, cell-patterning and robotics [9-12].

Differentiation of skeletal muscle is a highly controlled multi-step process, during which single muscle cells initially freely divide and then align and fuse to form multi-nucleated myotubes. This process of muscle differentiation *in vivo* is governed by a complex interplay of a wide range of growth factors and trophic factors. Several such factors have been discovered to date which have been observed to promote muscle differentiation *in vivo* [6, 7]. However, very little systematic research has been undertaken to use this extensive *in vivo* knowledge of growth factors to develop a chemically defined medium, without serum, which promotes muscle differentiation *in vitro*.

Most of the existing *in vitro* culture methods rely on the use of animal serum and biological growth substrates to promote skeletal muscle differentiation [13]. The presence of many unknown components in serum-containing medium, and the technical difficulties in creating reproducible biological substrates, has led to extensive variations in results from experiment to experiment. In order to remove this inherent drawback of serum containing medium and biological substrates, we attempted to develop a defined culture system consisting of a novel serum-free medium based on the extensive *in vivo* knowledge of growth factors and a synthetic silane substrate to study skeletal

muscle differentiation.

This work reports the development of such an *in vitro* cell culture system to study fetal rat skeletal muscle differentiation. The system had three novel features with respect to all other previously reported systems. First, a chemically defined, serum-free medium supplemented with specific growth factors was developed to study the muscle differentiation process. Second, a synthetic, non-biological, patternable, cell growth promoting substrate, N-1[3-(tri-methoxysilyl) propyl] diethylenetriamine (DETA) coated on glass coverslips, was used to grow the skeletal muscle cells. Third, we demonstrated that when the dissociated muscle cells were plated on fabricated microcantilevers, they aligned along the long axis of the cantilever and subsequently formed myotubes.

This *in vitro* system successfully demonstrated the differentiation of dissociated skeletal muscle cells (obtained from the hind limb of embryonic rat fetus) to form robust, contracting, multi-nucleated myotubes. These myotubes were further characterized by morphological analysis, using fetal myosin heavy chain and alpha-sarcomeric actin antibodies and electrophysiology. This novel system would not only find applications in skeletal muscle differentiation studies and biocompatibility studies but also in bioartificial muscle engineering, hybrid actuation system development, biorobotics as well as a defined test bed for better understanding of myopathies and neuromuscular disorders.

Methods and Materials

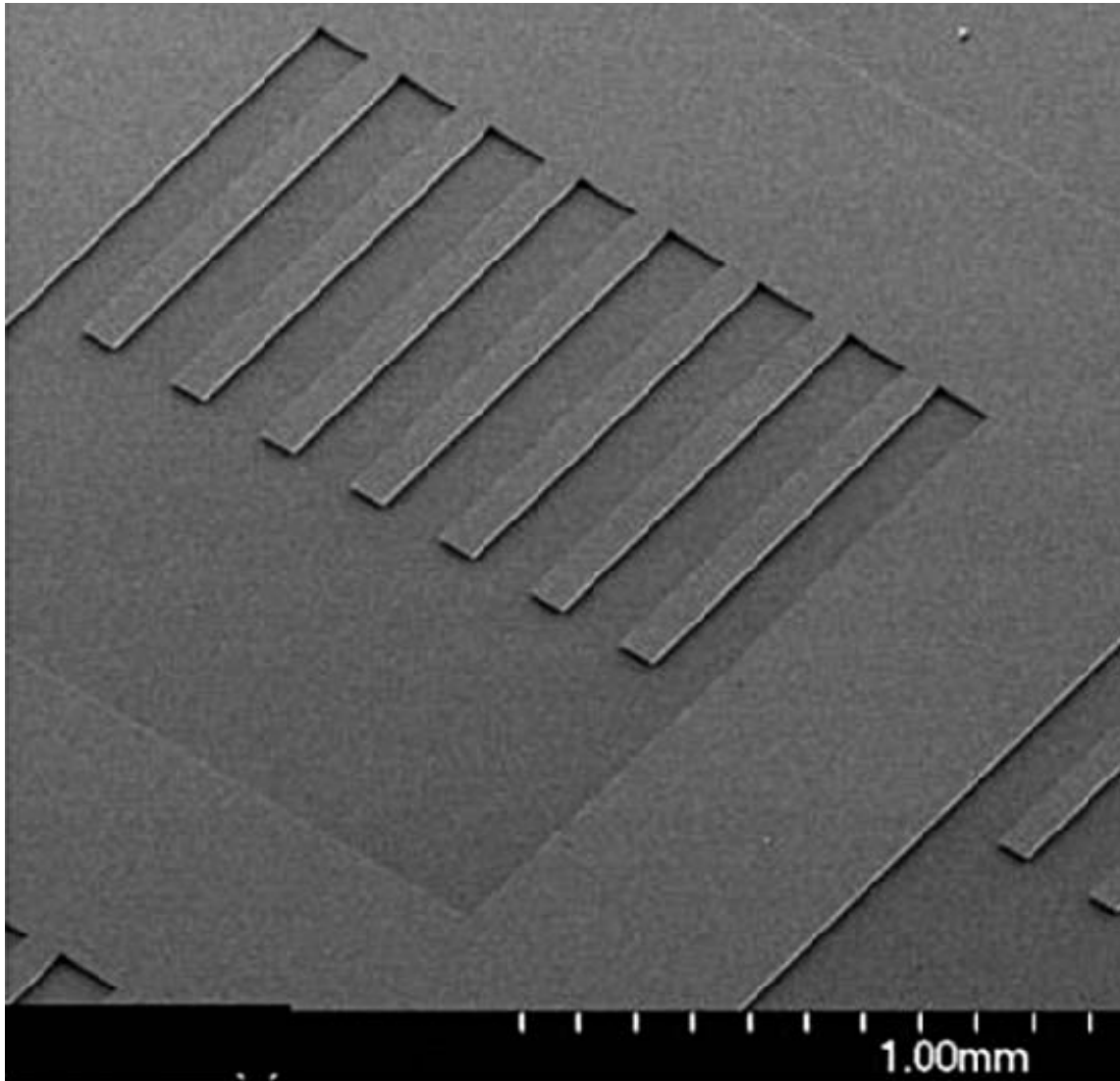
Surface Modification

Glass coverslips (Thomas Scientific 6661F52, 22x22mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) film was formed by reaction of the cleaned surface with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (Kratos Axis 165) by monitoring the N 1s peak.

Cantilever Fabrication

The fabrication process for the devices in Figure 7 and 10 was straight-forward. The design for the cantilevers was generated using AutoCAD 2004, and was used to create the photomask which consisted of a fused silica wafer coated with chromium. The cantilevers were fabricated from crystalline silicon wafers using a deep reactive ion etching (DRIE) process. A double-sided polished 10 μm thick crystalline silicon wafer was bonded to a 500 μm SiO₂ handle wafer and the crystalline silicon surface was coated with a 1.3 μm layer of AZ 5214 photoresist. The photoresist was exposed to a soft bake followed by contact exposure with the mask. The photoresist was then developed, hard baked, and then mounted on a 6" handling substrate for DRIE. After DRIE, the

photoresist was removed via a wet strip followed by plasma cleaning. The wafer was cut into 10 mm x 10 mm pieces, contained the cantilever arrays, by dicing followed by HF release and supercritical CO₂ drying. The processing was done through the MEMS Exchange of Reston, VA. A rectangular cantilever was used so that the spring constants could be easily calculated and it was hoped that the shape would aid and direct in the adhesion of the myotubes. The stated dimensions were chosen based partly on previous observations of average myotube size in culture and partly on the tolerances dictated by the current fabrication process.



S4700 5.0kV 10.8mm x 50 SE(L)

Figure 7. SEM micrograph of the fabricated cantilever.

Muscle Cell Isolation and Culture

The skeletal muscle was dissected from the thighs of the hind limbs of embryonic rat fetus at the indicated age (embryonic rat ages E15, E16 and fetal rat ages E17, E18). The tissue was collected in a sterile 15-ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 1 ml of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath (100 rpm). After 30 minutes the trypsin solution was removed and 2 ml of L15 + 10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated. The supernatant was then transferred to a 15-ml centrifuge tube. The same process was repeated two times by adding 2 ml of L15 + 10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300 g for 10 minutes at 4°C. The pellet obtained was resuspended in 1 ml of serum-free medium and plated in 100 mm uncoated dishes for 30-45 minutes. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and either plated directly or further processed using an additional purification protocol (see below). The cells were plated at a density of 700-1000 cells/mm². The cells attached to the substrate in 1 hour. The serum-free medium was added to the culture dish after 1 hour and the cells were maintained in a 5% CO₂ incubator (relative humidity 85%).

For E18 cells, we followed an additional purification protocol consisting of plating the cells in 100 mm dishes coated with a 2% gelatin solution (Type B from bovine skin, Sigma G1393) in serum-free medium and maintained them for 30-48 hours in a 5% CO₂ incubator. After 30-48 hours, spindle-shaped myoblasts were detached with neutral protease (0.6 unit/ml Dispase II, Roche 92517500) treatment for 2-5 minutes [13], then centrifuged on a BSA cushion, and replated onto the DETA coverslips.

Myotube yield quantification

Myotube yield was quantified using the fusion index, which is defined as the number of nuclei contained in the myotubes divided by the total number of nuclei counted in a given microscope field. Cultures, which were used for immunostaining, were simultaneously counterstained with 4',6-Diamidino-2-phenylindole (DAPI) which is a classic fluorescent nuclear and chromosome counterstain, and was used to identify nuclei and show the chromosome-banding patterns. DAPI binds selectively to dsDNA and thus shows little to no background staining of the cytoplasm. Nuclei were counted in 20 randomly chosen microscope fields from at least six separate muscle culture experiments.

Immunocytochemistry

Embryonic myosin heavy chain: Coverslips were rinsed with PBS, fixed in -20°C methanol for 5-7 minutes, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin

(permeabilization solution), and blocked for 30 minutes with 10% goat serum and 1% BSA. Cells were incubated overnight with primary antibody against embryonic MHC (F1.652, IgG, Developmental Studies Hybridoma Bank) diluted (1:5) in the permeabilization solution. Cells were washed with PBS and incubated with the secondary antibody (Cy3 conjugated anti-mouse, Jackson Labs., 1:200 dilution in PBS) for 2 hours[14].

α -actin: Coverslips were rinsed in PBS, fixed in 100% cold ethanol for 30 minutes, and rinsed again in PBS. The cultures were then blocked using 5% BSA (Sigma) in PBS for two hours. The primary antibody (mouse anti- α -actin, Sigma A2172, 1:800 dilution in blocking solution) was added for 12 hours at 4°C. The secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse, Molecular Probes, A21202, 1:200 dilution in PBS) was then added to the cultures for two hours. After a final rinse with PBS, the coverslips were mounted with Citifluor-mounting solution (Ted Pella) onto slides (Fisher). The coverslips were visualized using a Zeiss LSM 510 confocal microscope.

Electrophysiology

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Leibovitz medium, 35°C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with

intracellular solution (in mM: K-gluconate 140, EGTA 1, MgCl₂ 2, Na₂ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg, Hepes 10; Ph = 7.2). The resistance of the electrodes was 6-8 Mohm. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were performed with pClamp 8 software (Axon). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp 8 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from -85 mV to -95 mV without any whole-cell or series resistance compensation. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -85 mV holding potential. Action potentials were evoked with 1s depolarizing current injections from a -85 mV holding potential.

Results and Discussion

Surface Modification and Characterization

In the present study we used glass coverslips coated with DETA. The modified surfaces were analyzed by contact angle and X-ray photoelectron spectroscopy (XPS). XPS has previously been shown to be a good quantitative indicator of monolayer formation [1, 3, 15-17]. The contact angle and XPS data indicated that the glass surfaces were covered by a complete monolayer of DETA.

The first step in creating this defined system was to develop a synthetic surface to control cell-substrate interactions. The current biological substrates (collagen, gelatin, fibronectin) offer little hope to create quantifiable cell-substrate interactions with systematic modifications [13]. It was also our objective that our surface modification method should be integratable with silicon microstructures, compatible with surface patterning methods (stamping and photolithography) and it should enable relatively high throughput and flexible production of functionalized surfaces [1, 3]. Coating surfaces with self-assembled monolayers (SAMs) is a flexible and effective method to engineer surface characteristics of materials [18]. It has been shown that biological molecules can be incorporated into SAMs through crosslinkers, which could also enable selective study of specific contact signaling pathways [19]. SAM coated surfaces have also been used to grow and pattern hippocampal neurons, adult spinal cord neurons, motoneurons, cardiomyocytes, endothelial cells and muscle cell lines [2-4, 15, 19-22].

We chose DETA as the synthetic culture surface in our pilot study. Our earlier experiments proved that DETA is an appropriate surface to grow and pattern neurons [1, 3, 4, 16, 22] and endothelial cells [15], and it is our hope to integrate the motoneuron and skeletal muscle culture by having a common surface modification.

The cell-attachment promoting feature of DETA is possibly a result of its hydrophilic properties and the presence of a primary amine group as indicated in earlier publications for neurons and endothelial cells [1, 3, 4, 15-17, 22].

Development of Serum-Free Defined Medium

Our next aim was to formulate a defined medium which promoted muscle cell survival and differentiation on the DETA synthetic surface. The empirically-developed serum-free medium utilized our extensive experience in culturing mammalian cellular systems (Table 3). The basal medium consisted of L15 and Medium 199 in a 3:1 ratio. The B27 supplement and four growth factors (FGF-2, Cardiotrophin-1, GDNF, BDNF) were added to the medium. B27 is an optimized serum-free medium substitute generally used for neuronal culture. It consists of 27 different components, which include lipids, vitamins, hormones, antioxidants, and a few other miscellaneous components [23]. One of the B27 components is retinoic acid, which plays a key role in muscle differentiation [24]. Basic fibroblast growth factor (FGF-2) is a 17-kDa member of the heparin binding growth factors [25]. It plays a complex yet poorly understood role in muscle differentiation.

On one hand it stimulates limb development [26], but on the other hand tissue culture studies indicate that it is a mitogen and inhibits muscle differentiation [27]. In our study we found that even in the presence of basic FGF, the myocytes differentiate to form myotubes. Cardiotrophin-1 is a cytokine belonging to the IL-6 family. It is expressed at high levels in embryonic limb bud development and is secreted by differentiated myotubes [28, 29]. It is a potent cardiac survival factor and supports long-term survival of spinal motoneurons [30]. Glial cell line derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-beta superfamily [31]. GDNF plays a role in the differentiation and survival of central and peripheral neurons and in kidney organogenesis. GDNF is widely expressed in developing skeletal muscle [32]. Brain-derived neurotrophic factor (BDNF) is a ligand for the low-affinity NGF receptor, p75, and for the high-affinity neurotrophin receptor, trkB. It is expressed in developing skeletal muscle, promotes motoneuron survival and also plays a vital role in the formation of the neuromuscular junction [33, 34].

Table 3: Composition of the Serum-Free Medium for a 500ml Sample

Component	Source	Catalogue #	Amount
Leibovitz Medium (L15)	Invitrogen	11415064	375 ml
Medium 199	Invitrogen	11150059	125 ml
B27 Supplement (50x)	Invitrogen	17504044	10 ml
Basic Fibroblast Growth Factor	Invitrogen	13256029	10ng/ml
Brain Derived Neurotrophic Factor	Invitrogen	10908019	1ng/ml
Glial Derived Neurotrophic Factor	Invitrogen	10907012	1ng/ml
Cardiotrophin-1	Cell Sciences	CRC700B	10ng/ml
Sodium Bicarbonate	Fisher	5233500	0.93 mg
Osmolarity			320-325 mOsm
pH			7.3

Skeletal Muscle Culture

We used primary cultures of embryonic rat skeletal muscle at four different embryonic stages of rat development for the experiments. Mononucleated muscle cells were obtained by trypsinizing the hind limb muscle obtained from 14, 15, 17 and 18-day old rat embryos (E14, E15, E17 and E18). We selected these ages because all of the genes involved with limb development are expressed by the E14 stage [8]. In all experiments the cells were plated at a density of 700-1000 cells/mm [3]. Muscle cells obtained from the limb-bud of E14 and E15 fetuses formed only small, spindle shaped contractile structures (consisting of 2-3 nuclei). When we obtained the muscle cells from E18 fetuses, multinucleated myotubes were formed on top of a monolayer of other contaminating cells (mostly fibroblasts, Figure 8. A). In order to remove the fibroblast contamination we used a two-step panning protocol [13, 35]. After this purification step, myotubes were formed in direct contact with the DETA surface (Figure 8. B). Preparing the rat skeletal muscle cultures from 17-day old embryos resulted in a relatively pure culture (Figure 8. C, D). We obtained similar results using purified E18 (n = 5) or non-purified E17 (n = 20) rat skeletal muscle cultures. Our experiments demonstrated that DETA coated surfaces not only allowed the skeletal muscle cells to differentiate and promoted myotube formation, but also enabled them to maintain their contractile properties in the new medium formulation.

Multinucleated myotubes first appeared at the beginning of the second day of culture. Spontaneous contractions of the myotubes were observed by the end of the second day. Purified E18 and non-purified E17 myotubes were cultured to day 5.

In the E17 cultures, myoblast fusion began after 24 hours in the defined system and after 36-48 hours maximal fusion was reached ($59\% \pm 5.7$, mean \pm SD, of the nuclei were in the myotubes, averaged from 10 separate cultures, $n = 10$). In the E18 double step-purified culture, myoblast fusion began after 36 hours in the defined system and after 96 hours reached maximal fusion ($36\% \pm 5.5$, mean \pm SD, of the nuclei were in the myotubes, averaged from 6 separate cultures, $n = 6$).

Immunocytochemistry

Immunocytochemistry combined with confocal microscopy and electrophysiological methods were used to characterize the myotubes. Myotubes were labeled by skeletal muscle markers for α -actin and the embryonic myosin heavy chain [14], to facilitate unambiguous identification (Figure 8. E, F).

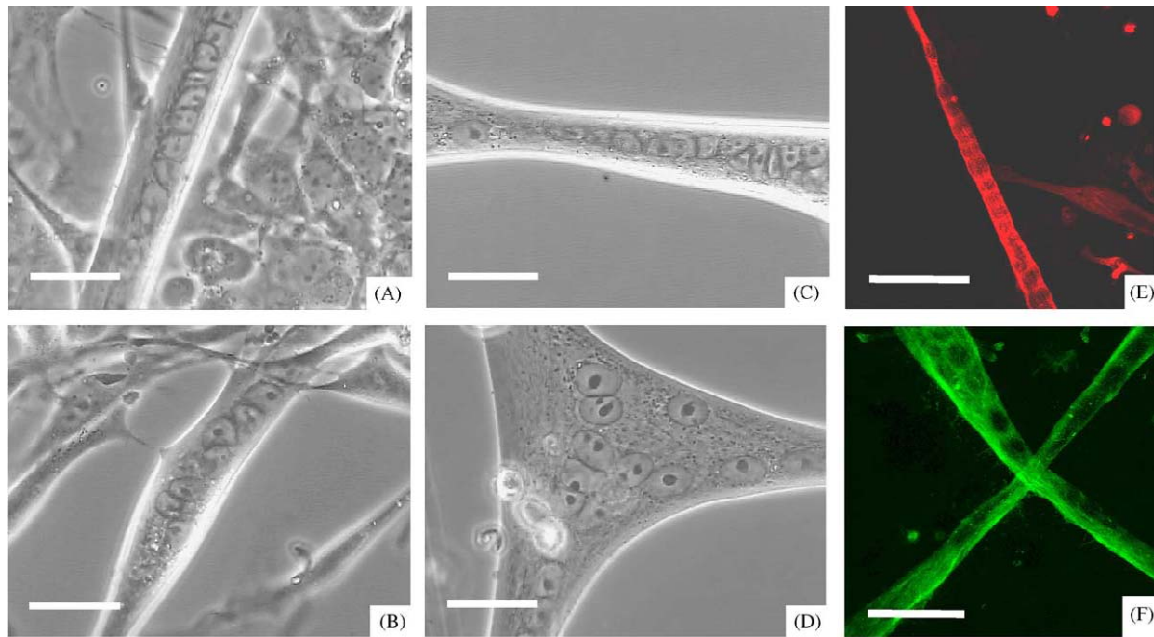


Figure 8. Representative pictures of myotubes formed in our defined system at day 4 and 5. A: E18 non-purified culture. Myotubes were formed on the top of a monolayer of other cell types. B: Myotubes in E18 purified cultures. C, D: Multinucleated myotubes in E17 cultures. E: Myotubes immunostained for myosin heavy chain. F: Myotubes immunostained for α -actin. A, B, C, D: Phase contrast 40x, scalebar: 25 μ m. E: Confocal 40x, scalebar: 75 μ m. F: Confocal 63x, scalebar: 40 μ m.

Electrophysiology

Patch-clamp recordings were obtained from the contractile myotubes on the 4th day in vitro (Figure 9) [36]. The large membrane capacitance of the myotubes indicated that they consisted of many fused muscle cells. Voltage-clamp recordings showed the presence of voltage dependent inward and outward currents in the cell membrane that are consistent with sodium and potassium channels, respectively. In the current clamp recordings most of the myotubes were able to generate action potentials. Upon stimulation (depolarization) all recorded myotubes demonstrated contraction. Some myotubes showed spontaneous contractions after the medium was changed. There was a significant difference in the electrophysiological parameters between E18 non-purified and E18 purified /E17 cultures. In E18 non-purified cultures the membrane potential was more negative (-61 ± 1.8 mV, mean \pm SEM, n = 6) compared to the pure cultures (-49.4 ± 1.7 mV, n = 8). The membrane capacitance of the myotubes was significantly higher in the non-purified cultures (1349 ± 241 pF) compared to the pure cultures (566 ± 177 pF). This indicates that the average size of the myotubes in the non-purified cultures was larger. This points out that there may be some influence from the fibroblasts in myotube differentiation. In all the electrophysiological recordings from the myotubes, independent of the purity of the culture, there were voltage-dependent inward and outward currents present (Figure 9). The electrophysiology results indicate the formation of robust, functional myotubes in our defined system that would have the correct properties to serve as actuators in hybrid systems.

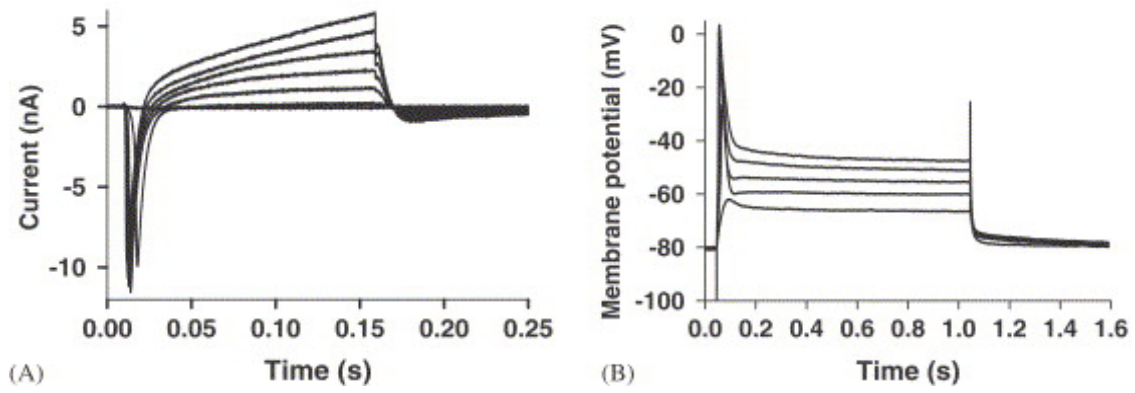


Figure 9. Representative electrophysiological recordings obtained from 4-day-old myotubes. (A) Voltage-clamp experiments indicated that myotubes formed on a DETA surface in the serum-free medium formulation expressed functional voltage-dependent sodium and potassium channels. (B) Current-clamp mode depolarization with evoked action potentials, which were associated with visible contractions.

Myotube on Silicon Microstructures

In ten different experiments, we plated the dissociated muscle cells (obtained from E17 rat fetus) on unpatterned, DETA-coated microcantilevers (Fig. 7). In 50% of the cantilevers from each experiment, we observed that dissociated muscle cells aligned along the long axis of the cantilever to form contracting myotubes. It should be noted that no discernable preference was found for any particular dimension set. While the myotubes were observed to be contractile, it was not possible to visually confirm that they were bending the cantilevers. This is due to the fact that the spring constants of the cantilever were too high compared to the contractive strength of the myotubes. However, it cannot be said that the cantilevers did not bend at all, because no measurements sensitive enough to detect such a deflection were performed. These experiments have been reserved for future work. At this point we feel that the investigations of the myotubes integrated with the microstructures, as indicated in Fig. 10, now facilitate the future development of hybrid actuation systems for applications in biorobotics, prosthesis and bioartificial muscle engineering.

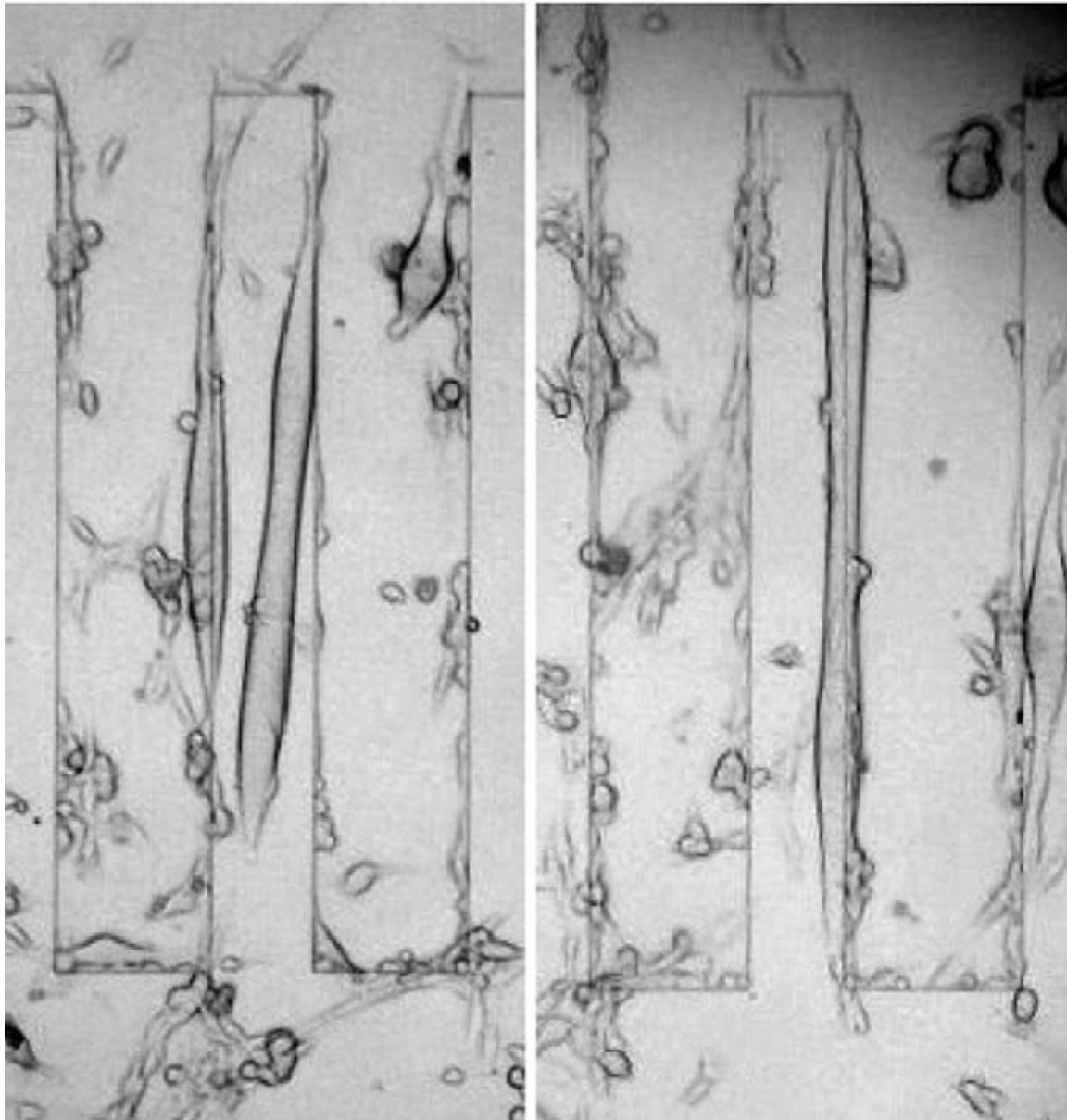


Figure 10. Myotubes forming on fabricated microcantilevers in serum-free medium in two separate experiments (middle and right). *Note:* the myotubes generally lined up with the long axis of the cantilever.

In conclusion, in this study we have developed a defined system (synthetic substrate, serum-free medium and specific cellular preparation) which promotes muscle differentiation and functional myotube formation. Using this approach, the myotubes could be easily integrated with silicon-based microstructures, and used in model systems, specifically the reflex arc, or as actuators in hybrid systems. Our serum-free medium and defined system, we believe, will become essential for tissue engineering and biocompatibility studies. It is also compatible with surface patterning methods, and therefore it will enhance the integration of our muscle constructs with microelectromechanical systems (MEMS) to create hybrid devices for robotic and prosthetic applications. These are the first studies whereby simultaneously controlling both the growth substrate and the composition of the medium, the differentiation of the myocytes could be manipulated and one can now consider the idea of evolving functional hybrid materials from progenitor cells[5].

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CHAPTER 4: DIFFERENTIATION OF SKELETAL MUSCLE AND INTEGRATION OF MYOTUBES WITH SILICON MICROSTRUCTURES USING SERUM-FREE MEDIUM AND A SYNTHETIC SILANE. NATURE PROTOCOLS 2007;2(7):1795-801

Introduction

This protocol describes a cell culture model to study the differentiation of fetal rat skeletal muscle cells. The model uses serum-free medium, a non-biological substrate (N-[3 (trimethoxysilyl) propyl] diethylenetriamine; DETA) and fabricated microcantilevers to promote the differentiation of dissociated rat myocytes into robust myotubes. In this protocol we also describe how to characterize the myotubes based on morphology, immunocytochemistry and electrophysiology. Four major techniques are employed: fabrication of cantilevers; surface modification of the glass and cantilever substrates with a DETA SAM; a serum-free medium and refined culture techniques. This culture system has potential applications in biocompatibility studies, bioartificial muscle engineering, skeletal muscle differentiation studies and for better understanding of myopathies and neuromuscular disorders. The model can be established in 26-33 days.

Differentiation of skeletal muscle is a highly-controlled, multi-step process, during which single muscle cells initially freely divide and then align and fuse to form multinucleated myotubes. This process of muscle differentiation in vivo is governed by a complex interplay of a wide range of growth and trophic factors. Several such factors have been discovered which have been observed to promote muscle differentiation in vivo[1-3]. However, very little systematic research efforts have

been undertaken to use this extensive in vivo knowledge of growth factors to develop a chemically defined medium which promotes muscle differentiation in vitro.

Most of the existing in vitro culture methods for studying skeletal muscle differentiation use serum containing medium as well as a biological growth substrate[4-6]. The presence of many unknown components in serum-containing medium and the technical difficulties in creating reproducible biological substrates has led to extensive variations in the results from experiment to experiment. In order to remove the inherent drawback of utilizing serum containing medium and biological substrates, a defined culture system consisting of a serum-free medium based on the extensive knowledge of growth factors from in vivo studies and a synthetic silane substrate to study skeletal muscle differentiation, was developed[7].

The system has three features that differentiate it from all other previously reported systems. First, a unique chemically defined serum-free medium supplemented with specific growth factors was developed to study the muscle differentiation process. Second, a synthetic, non-biological, patternable[8, 9], cell growth promoting substrate, N-1[3 (trimethoxysilyl) propyl] diethylenetriamine (DETA), coated on glass coverslips[10-12], was used as a template to grow the skeletal muscle cells. Third, it was demonstrated that when the dissociated muscle cells were plated on fabricated microcantilevers, they aligned along the long axis of the cantilever to form aligned myotubes.

In this protocol step-by-step instructions on how to fabricate and modify the cantilever and glass substrates and then grow the muscle cells on the chemically modified substrates, using this serum-free medium, to form robust myotubes, is described. Figure 11 shows a diagram of the general outline of the whole procedure, while Figure 12 outlines the microcantilever manufacture process.

Figure 11. General outline of the entire procedure.

Note that all fabrication procedures should be performed in a clean room. The protocol consists of two figures: Figure 13, which shows a microcantilever array micrograph, and Figure 14, which outlines the cell culture process; as well as substrate modification of either a cantilever or a glass substrate (Steps 1-22), preparation of the serum-free medium, as well as both intracellular and extracellular solutions (Reagent Setup), dissection of the skeletal muscle tissue (Steps 23-24), purification of the tissue and the culture of the myocytes to form myotubes (Steps 25-38), and characterization of the myotubes morphologically, immunocytochemically and electrophysiologically (Steps 39-40 A,B,C).

Materials and Methods

List of Reagents

Charles River or Sprague Dawley, Fetal Rats age E18.

Caution: Experiments involving live animals must conform to appropriate National and Institutional regulations

*H₂SO₄ (EMD)

*HCl (EMD)

* Caution toxic and corrosive. Use gloves, goggles and other chemical use safety measures that are recommended by the appropriate concerned agencies.

Countertop oven (VWR)

Toluene (BDH) Caution toxic

Hydrofluoric acid (Sigma) Caution Toxic

Diethylenetriamine trimethoxysilane (United Chemical Technologies)

4 inch silicon on insulator (SOI) wafers (MXF)

AZ 5214E photoresist (Clariant Corporation)

AZ 9245E photoresist (Clariant Corporation)

AZ 400K photoresist developer (Clariant Corporation)

Deep reactive ion etcher (PlasmaTherm)

Ethanol

4',6-Diamidino-2-phenylindole (DAPI)

Leibovitz medium (L15)Invitrogencat no. 11415064

Medium 199Invitrogencat no. 11150059

B27 Supplement (50×)Invitrogencat no. 17504044

Basic fibroblast growth factor (b-FGF)Invitrogencat no. 13256029

Brain-derived neurotrophic factor (BDNF)Invitrogencat no. 10908019

Glial-derived neurotrophic factor (GDNF)Invitrogencat no. 10907012

Cardiotrophin-1 (CT-1)Cell sciences cat no. CRC700B

Sodium bicarbonate Fishercat no. 5233500

Trypsin EDTA

Phosphate-buffered saline (PBS)

Fetal calf serum (FBS)

Bovine serum albumin (BSA)

Embryonic MHC (F1.652, IgG, Developmental Studies Hybridoma Bank)

Primary antibody (mouse anti- α -actin, Sigma A2172)

Secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse Molecular Probes, A21202)

Citiflour-mounting solution (Ted Pella)

Clean glass coverslips (Thomas Scientific 6661F52, 22×22 mm No. 1)

O₂ plasma cleaner (Harrick PDC-32G)

DETA (United Chemical Technologies Inc. T2910KG)

0.1% (v/v) mixture of organosilane in freshly distilled toluene (Fisher T2904).

Phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075).

Trypsin-EDTA (Gibco 25300054)

Fetal calf serum (FBS) (Gibco 16000044)

L15 medium Invitrogen (Cat # 41300021).

BSA (Sigma A3059)

Alexa Fluor 488-conjugated donkey anti-mouse antibody, Molecular Probes, A21202, for immunocytochemistry only

Mouse anti- α -actin, Sigma cat no A2172, for immunocytochemistry only

Borosilicate glass pipettes (BF150-86-10, Sutter), for electrophysiology only

HEPES (Fisher, BP310), for electrophysiology only

HEPES-sodium (Fisher, BP410), for electrophysiology only

EGTA (Fisher, O2783), for electrophysiology only

K-Gluconate (Sigma, G4500), for electrophysiology only

MgCl₂ (Fisher, S320), for electrophysiology only

Na₂ATP (Fisher, BP413), for electrophysiology only (CRITICAL Keep at -20°C)

Secondary antibody against embryonic MHC (F1.652, IgG, Developmental Studies Hybridoma Bank)

List of Equipment

Pyrex bottles (VWR)

Pyrex beakers (VWR)

Hotplate (VWR)

Ceramic racks (VWR)

Glove Box (MBraun)

Thermometer (VWR)

Dissecting instruments

Dissecting hood

Laminar flow hood

Microscope – regular and confocal

Carbon dioxide incubator

Pasteur pipette

Contact angle goniometer (KSV Instruments, Cam 200 (or similar instrument))

X-ray Photoelectron Spectrometer (Kratos Axis 165 (or similar instrument))

Axioscope 2 FS Plus upright microscope (Zeiss, Jena, Germany), for electrophysiology only

PCS-5000 piezoelectric 3D micromanipulator (Burleigh/EXFO, Quebec, Canada), for electrophysiology only

Gibraltar platform (Burleigh EXFO, Quebec, Canada), for electrophysiology only

Vibration isolation table (TMC, Peabody, MA, USA), for electrophysiology only

Multiclamp 700B patch clamp amplifier (Axon/Molecular Devices Sunnyvale, CA, USA), for electrophysiology only

Digidata 1320 A/D converter (Axon/Molecular Devices Sunnyvale, CA, USA), for electrophysiology only

pClamp 9.0 software (Axon/Molecular Devices Sunnyvale, CA, USA), for electrophysiology only

P97 pipette puller (Sutter, Novato, CA), for electrophysiology only

In-line solution heater (self-made but available from Warner Instruments, Hamden, CT, USA), for electrophysiology only

pH meter (Beckman), Balance (Mettler-Toledo), Osmometer (Fiske), for electrophysiology only

Reagent Setup

Serum-free medium

Combine the 375 ml Leibovitz medium (L15), 125 ml Medium 199, 10 ml B27 Supplement (50X), 10 ng/ml of basic fibroblast growth factor (B-FGF), 1 ng/ml of brain derived neurotrophic factor (BDNF), 1 ng/ml of glial derived neurotrophic factor (GDNF), 10 ng/ml of cardiotrophin-1 (CT-1) and 0.70 g of sodium bicarbonate. Adjust the pH of the medium to 7.3 with 1N NaOH and the osmolarity to 320-325 mOsm with D.V. The components can be added all at once, in no particular order. Store in 50 ml sterile polycarbonate tubes at 4°C. The medium will last 2 months.

Intracellular solution

Combine 0.15ml HEPES-sodium salt (from a 1M solution), 11.4mg EGTA, 983.6mg K-Gluconate, 0.3ml $MgCl_2$ (from a 200mM stock solution), 30.7mg Na_2ATP and 30ml deionized water. Adjust the pH to 7.2 with HEPES, and adjust the osmolarity to 276 mOsm, with D.V.

Critical: The intracellular solution should be prepared in the given order. EGTA dissolves only at basic pH. Store the aliquot intracellular solution in 1 ml freezing vials at $-20^{\circ}C$.

Extracellular solution

Take 50 ml of the Leibovitz medium (L15), check the pH and adjust to 7.34 with HEPES, also adjust the osmolarity to 320 mOsm with D.V.

Procedure

Microcantilever fabrication and surface modification of microcantilevers and glass coverslips.

Timing 2-3 weeks

1) Fabrication of microcantilevers (Figures 12 and 13). Start procedure with a 4" double-sided polished silicon-on-insulator (SOI) wafers that contain a 5 mm thick top layer of crystalline silicon bonded onto a 500 nm thick silicon dioxide layer.

Figure 12. Outline of microcantilever manufacture procedures.

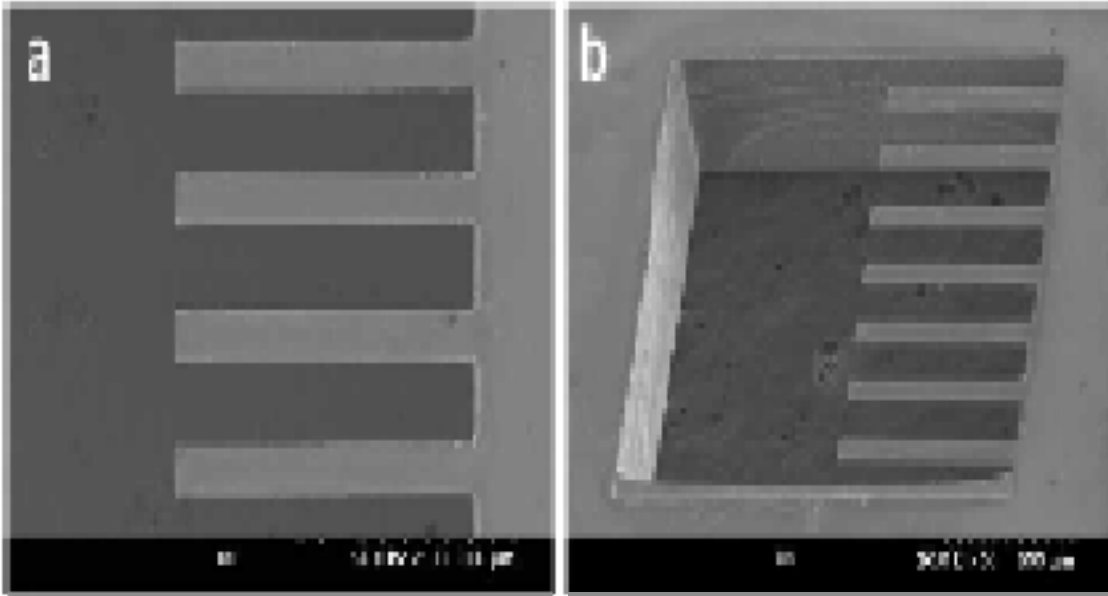


Figure 13. SEM micrographs of microcantilever array. (a) Top down view of cantilevers. (b) 45° angle view of cantilever array.

Critical step: All fabrication procedures must be performed in the clean room.

2) Perform photoresist spinning and developing in a Suss ACS200 wafer coater/developer (or similar instrument). First, prime the device layer (5 mm silicon) with a 100 Å layer of hexamethyldisilazane (HMDS). Then place the wafer, silicon layer up, on a resist spinner vacuum holder. Center the wafer and apply the AZ5214e photoresist to the center of the wafer. Begin spinning the wafer to spread the resist to a final thickness of 2.1 μm (spin at 1900 rpm).

3) Bake the resist at 110°C for 50 minutes.

4) Expose the wafer on a Suss MA6 aligner to the photomask containing the pattern for the cantilever. After exposure, develop the resist in a 1:1 mixture of AZ320 and deionized water.

5) Perform a Deep Reactive Ion Etching (DRIE) in a PlasmaTherm 770 DRIE etcher (or similar instrument) at a rate of 4 μm/min. Etch the 5 mm device layer through to the silicon dioxide layer. Remove the photoresist using a Metroliner M4L Plasma Resist Etcher (or similar instrument).

Troubleshooting

6) Flip the wafer over and prime the silicon dioxide layer with a 100 Å layer of HMDS. Next coat with AZ9245 photoresist to a final thickness of 10 μm (1000 rpm) and softbake at 100°C for 2 minutes. Place the coated wafer in a Suss MA6 Aligner (or similar instrument) and expose the resist to the mask containing the pattern for the back side pattern. Note: The Suss MA6 Aligner is capable of front/back alignment. For this process it is necessary to incorporate the alignment marks in the photomasks.

7) After exposure immerse the wafer in AZ 400K developer to develop the patterned resist. This is followed by a hard bake at 120°C for 30 minutes. Etch until approximately 50um etch depth is left.

8) Mount the wafer on a dummy wafer using Nitto Model 3195V thermal release tape. Complete the DRIE etching.

9) Demount the wafer by heating to 170°C on a hotplate. Remove the photoresist by plasma etching in a Metroline M4L Plasma Resist Etcher (or similar instrument). Perform the final etch in 49% HF for 10 minutes to remove the buried oxide layer (Caution: HF is extremely toxic).

Surface modification of the cantilevers with DETA Timing 2-3 days

10) Immerse the cantilever die in a 1:1 solution of absolute MeOH and concentrated HCl for 15-30 minutes, followed by a 3x wash in deionized water. Next, place the die in a bath of concentrated sulfuric acid for 30-45 minutes. Note that at this point you need to ensure that the die are not touching as they may stick together during this step.

11) Gently rinse the cantilever die in deionized water. Finally, boil the cantilever die in deionized water for 30 minutes. Then place the cantilevers in a 120°C oven overnight.

12) Prior to the surface modifications prepare all glassware and reagents for the reaction. Clean all glassware in a base bath (saturated KOH in 100% methanol) overnight and then rinse in deionized (18 MΩ resistance) water. Bake the glassware overnight in a 120°C oven. Distill Toluene over metallic sodium in order to ensure minimal water content. CRITICAL STEP This is necessary to

minimize the presence of water in the reaction mixture. Note that excess water causes the polymerization of the silane monomer.

13) Transfer the desired volume of toluene to a clean/dry (see step 2) Pyrex bottle. Before sealing the bottle, blow dry nitrogen into the unused volume in order to minimize the gaseous oxygen. Seal the bottle and place in the antechamber of the glove box. Evacuate the antechamber for 10 minutes and then vent with dry nitrogen, repeat 3 times. Transfer the toluene to the main chamber of the glove box.

14) Add stock DETA solution to the toluene for a final concentration of 0.1% (e.g. 0.2 ml in a final volume of 200 ml). Immediately remove the 0.1% DETA solution from the glove box and transfer into a glass beaker containing the cantilever die.

15) Place the reaction mixture on a hot plate and heat to $\sim 80^\circ$ for 30 minutes. After 30 minutes remove the reaction mixture from the hot plate and cool for 30 minutes or until the reaction mixture can be handled with bare hands.

16) Rinse the die 3 times in clean/dry toluene. Transfer the cantilever die into a fresh beaker and cover with distilled toluene.

17) Place the beaker back onto the hotplate and heat to $\sim 80^\circ\text{C}$ for 30 minutes. Finally, remove the cantilevers from the beaker and bake overnight at 120°C .

Cleaning glass coverslips and subsequent surface modification with DETA

18) Clean glass coverslips (22×22 mm No. 1) using an O_2 plasma cleaner for 20 min at 100 mTorr.

19) Form the DETA film by reacting the cleaned surface with a 0.1% (v/v) mixture of organosilane in freshly distilled toluene

20) Heat the DETA coated coverslips to just below the boiling point of the toluene, rinse with toluene, reheat to just below the boiling temperature, and then dry in an oven.

Characterizing the glass coverslips using contact angle measurements and X-ray photoelectron spectroscopy (XPS)

21) Characterize surfaces by contact angle measurements using an optical contact angle goniometer.

22) Use XPS to characterize the monolayer of DETA by monitoring the N 1s peak[9, 13].

Pausepoint: Samples should be stored in dessicator prior to use. DETA monolayers should be stable under anhydrous conditions for several weeks to months. Samples more than a couple months old should be reanalyzed to ensure the monolayer has not degraded.

Muscle Cell Isolation and Culture : Isolation of the myoblasts Timing 4-6 hrs (Figure 14)

23). Dissect the skeletal muscle from the hind limb thighs of a rat fetus at fetal rat age of E18.

24) Collect the tissue samples in a sterile 15-ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free).

25) Enzymatically dissociate the tissue samples using 3 ml of 0.05% of trypsin-EDTA solution for 60 minutes in a 37°C water bath, with agitation of 100 rpm.

26) After 60 minutes, remove the trypsin solution and add 6 ml of L15 containing 10% fetal calf serum (FBS) to terminate the trypsin action.

27) Mechanically triturate the tissue using a sterile narrow bore Pasteur pipette. Critical step: Please make sure not to form air bubbles, as formation of air bubbles damages the dissociating cells.

28) Allow the dissociated tissue to settle for 3 minutes.

29) Transfer the 6 ml supernatant to a 15-ml centrifuge tube.

30) Repeat the same process (steps 26-29) two times by adding 6 ml of L15 + 10% FBS each time.

31) Suspend the 18 ml cell suspension obtained after mechanical trituration on a 6 ml, 4% weight/volume (w/v) BSA (prepared in L15 medium) cushion and centrifuge at 300 g for 10 minutes at 4°C.

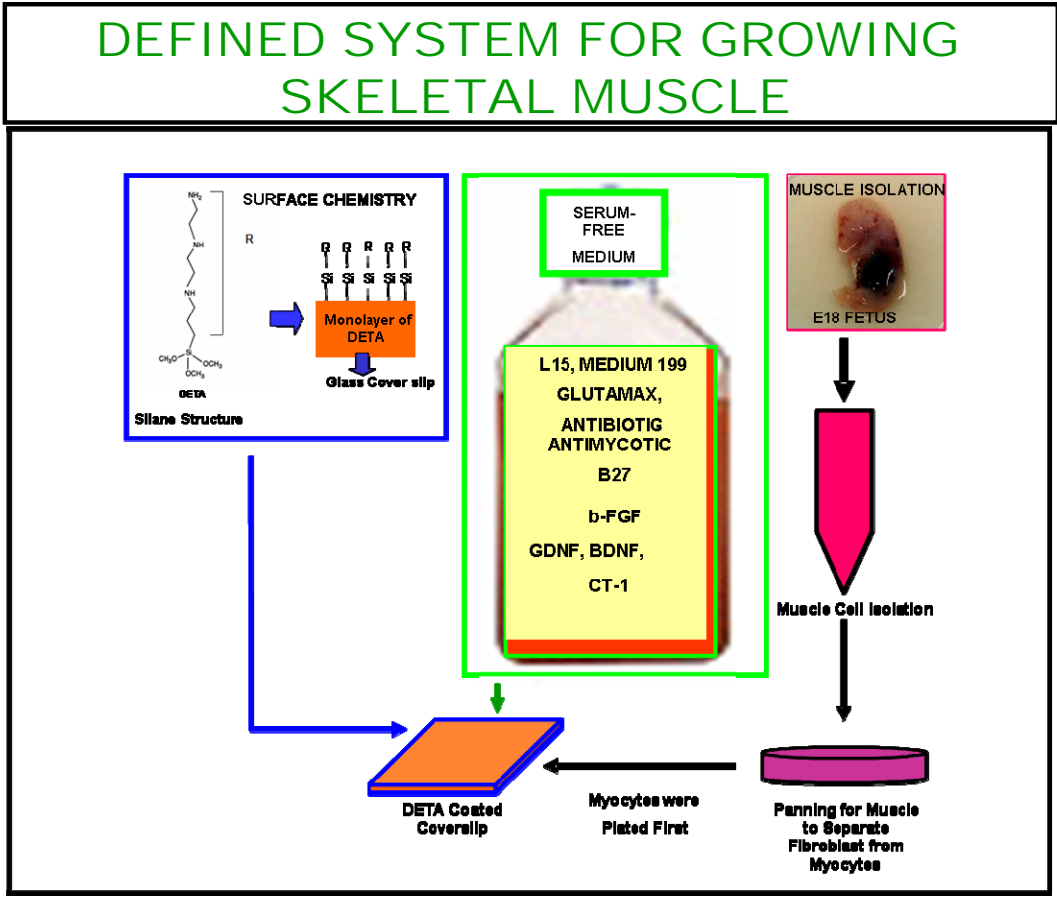


Figure 14. Defined system for growing skeletal muscle. Outline of the cell culture process.

32) Resuspend the obtained pellet in 10 ml L15 + 10% FBS and plate in 100 mm uncoated dishes for 20-30 minutes depending upon the amount of tissue. During this step, remove the contaminating fibroblasts. The fibroblasts should settle down and attach to the bottom of the 100 mm dish faster than the myocytes. The supernatant should contain pure myocytes.

33) Gently remove the non-attached cells that are present in the supernatant after 20-30 minutes by using a sterile Pasteur pipette.

34) Transfer the supernatant onto the top of a 6 ml 4% BSA cushion, and centrifuge at 300 g for 10 minutes at 4°C.

35) Resuspend the pellet in 1 ml of serum-free medium.

36) The cells are now ready for plating. At this point, conduct a cell count and plate the cells at a density of 500-800 cells/mm² on either coverslips or fabricated cantilevers.

37) Add the medium 1 hour after plating. Maintain the cultures in a 5% CO₂ incubator (relative humidity 85%).

38) Change half of the medium after every 4 days.

Refer to Table 4 for Troubleshooting Steps 23-28 as outlined above.

Characterization of the myotubes: Morphological quantification and fusion index

39) Quantify the myotube yield using the fusion index, which is defined as the number of nuclei contained in the myotubes divided by the total number of nuclei counted in a given microscope field.

40) The myotubes can now be immunostained for alpha-actin (option A) or embryonic myosin heavy chain antibody[5, 7] (option B)_or characterized using electrophysiology (option C).

A) Immunostaining myotubules for alpha-actin Timing 2 days

i) Rinse coverslips in PBS, fix in cold ethanol (100%) for 20 minutes, and rinse again in PBS.

ii) Block the cultures by incubating in 5% BSA in PBS for two hours at 4°C.

iii) Add the primary antibody (mouse anti- α -actin, 1:800 dilution in blocking solution) and incubate for 12 hours at 4°C.

iv) Then add the secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse, 1:200 dilution in PBS) to the cultures and incubate for two hours 37°C.

v) Rinse with PBS.

vi) Mount the coverslips with Citiflour-mounting solution onto slides.

vii) Visualise the coverslips, we use a Zeiss LSM 510 confocal microscope; a similar instrument could be used.

B) Immunostaining myotubes with the embryonic myosin heavy chain antibody:_ Timing 2 days

i) Rinse coverslips with PBS,

ii) Fix the cells in -20°C methanol for 5-7 minutes.

iii) After 5 minutes gently wash the coverslips in PBS in order to remove the excess fixing agent and then further incubate in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 5 minutes.

iv) Block the cells for 30 minutes with 10% goat serum and 1% BSA.

v) Incubate the cells overnight with the primary antibody against embryonic MHC (F1.652, IgG,) diluted (1:5) in the permeabilization solution.

vi) Wash the cells with PBS and incubate with the secondary antibody (Cy3 conjugated anti-mouse, 1:200 dilution in PBS) for 2 hours

vii) After incubating in the secondary antibody for 2 hoursm the coverslips are finally rinsed with PBS and then mount the coverslips with Citiflour-mounting solution onto slides. Visualize the coverslips using a confocal microscope or similar instrument. Cultures used for immunostaining are simultaneously counterstained with 4',6-Diamidino-2-phenylindole (DAPI), a classic fluorescent nuclear and chromosome counterstaining marker, and used to identify the nuclei and indicate the chromosome-banding patterns. DAPI selectively binds to dsDNA and, thus, shows little to no background staining in the cytoplasm. Immuno stained coverslips can be stored for more than one year. The counting is done at the time of fusion index analysis of the success of the culture system. Generally, we perform this analysis soon after preparing the coverslips. Count nuclei in 20 randomly chosen microscope fields from a minimum of six separate muscle culture experiments.

C) Electrophysiological characterization of the myotubes using patch-clamp electrophysiology

Timing 1 day

- i) Place the glass coverslips with the cultured skeletal muscle myotubes in a chamber on the microscope stage.
- ii) Continuously perfuse the chamber (2 ml/min) with the extracellular solution through the in-line heater (35°C) by gravity.
- iii) Pull a glass pipette (3 - step pull, Heat = Ramp Value, Pull = 0, Velocity = 30, Time = 200, Pressure = 300) with the electrode puller and fill with the intracellular solution. The electrode resistance should be 6-8 M Ω . Critical step: Electrode resistance can be slightly influenced by the Heat settings (± 10). If there are less or more than 3 steps, change the Velocity value (± 5). Electrode properties are highly dependent on the properties of the glass tube. We prefer to use a 3 mm wide box heating filament (more evenly heats) in the puller than the traditional u-shape filament.
- iv) Place the electrode into the headstage; apply positive pressure (about 2cc from a 10cc syringe) to the pipette before touching the extracellular solution.
- v) Bring the electrode close to the target cell; compensate for the pipette offset; touch a cell with the tip of the electrodes (seal position) under visual control.
- vi) Apply a -5 mV seal test; compensate pipette capacitance.
- vii) Apply a -70 mV holding (voltage clamp mode).
- viii) Form a gigaseal.

- ix) Rupture the cell membrane by the application of short suction pulses; measure resting membrane potential in $I = 0$ mode; compensate for whole cell capacitance and resistance.
- x) Set the Amplifier gain to 2 and apply a 2 kHz low-pass filter. Digitize signals at 20 kHz.
- xi) Correct all membrane potential data by subtracting a 15 mV tip potential. This is calculated using the Axon's pClamp 8 program.
- xii) Measure membrane resistance and capacitance using 50 ms voltage steps, from -85 mV to -95 mV, without any whole-cell or series resistance compensation followed by single-exponential fitting ($\tau = R_m C_m$).
- xiii) Measure the sodium and potassium currents in voltage clamp mode using 10 mV voltage steps from a -85 mV holding potential. Measure the sodium channel current amplitude at -25 mV, where no potassium channel activation is observed. Measure the potassium channel current at $+15$ mV at the end of the voltage step, when sodium channels are inactivated.
- ix) Evoke action potentials with 1s depolarizing current injections from a -85 mV holding potential.

Timing

From microcantilever fabrication through immunocytochemical and electrophysiological characterization:

Days 1-14/21 (2-3 weeks) Microcantilever fabrication (Steps 1-8)

Days 14-21 (2-3 days) Clean and coat the microcantilevers and glass coverslips with DETA (Steps 10-22)

Days 16-23 (4-6 hours) Cell culture including dissection, tissue isolation and plating (Steps 23-40)

Days 26-33 (1 day) electrophysiological characterization, (2 days) immunocytochemical characterization and (2 days) immunostaining myotubes for alpha-actin (Steps 40 A, B, C)

Table 4: Troubleshooting

Step	Problem	Possible cause	Solution
5	A cloudy film developing on the cantilever dice or on the glass coverslip	Polymerization of the DETA monomer; excess ambient humidity;	Make sure all excess water is removed from the reaction mixture; ensure glassware is properly cleaned and oven dried (120°C); ensure atmospheres are 60% relative humidity or less, if humidity is too high, perform the reaction in a controlled glovebox
23-24	Contamination of cells after plating	Contamination introduced prior to plating, possibly in the dissection	Prior to dissection spray instruments with ethanol (70%); perform dissection under a hood; change gloves between dissection and culture of the cells
25-27	Difficult trituration	Tissue was not digested enough	Confirm water bath temperature is 37°C; in regards to the enzymes – check the expiration date, confirm length of time in water bath before use (less than 1 hour)
25-27	Low yield of cells with unnecessary debris in culture	Over-trituration or excessive air during trituration	Use a larger-bore pipette; reduce bubbles created in the process by being more vigilant
25-27	Low yield of cells with tissue pieces remaining intact	Under-trituration	Use a smaller-bore pipette
23-38	Unhealthy cells after plating	Wrong pH of medium, wrong temperature/atmosphere in incubator; over-trituration or digestion	Verify medium pH adjust to 7.4 as necessary; verify incubator temperature and CO ₂ pressure (37°C and 5%); reduce trituration and digestion time

Results and Discussion

Glass coverslips and cantilevers coated with DETA are analyzed by contact angle and XPS. XPS has been shown to provide a good quantitative indicator of monolayer formation. The contact angle, along with XPS data, indicates that the surfaces are covered by a complete monolayer of DETA.

In the cultures, myoblast fusion begins after 24 h in the defined culture system and after 36–48 h maximal fusion is reached. Spontaneous contractions of the multi-nucleated myotubes are observed by the end of the second day (Figure 15).

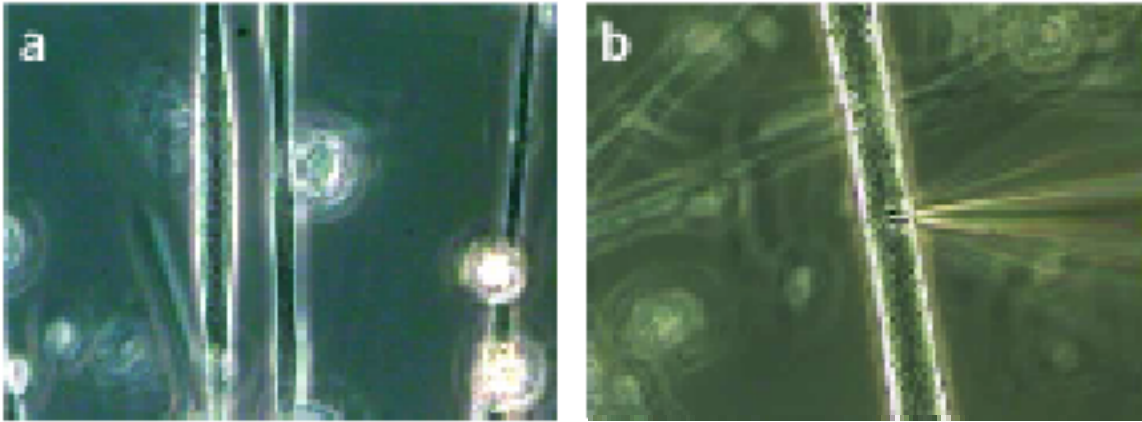


Figure 15. Phase pictures of myotubes. A. Phase picture of 7 day old myotubes. B. Phase picture of a 7 day old myotube with a patch-clamp electrode on its surface for electrophysiological studies.

Immunocytochemistry combined with confocal microscopy and electrophysiological methods are then used to characterize the myotubes. Myotubes are observed to be labeled by the skeletal muscle markers for α -actin and the embryonic myosin heavy chain, to facilitate unambiguous identification (Figure 16).

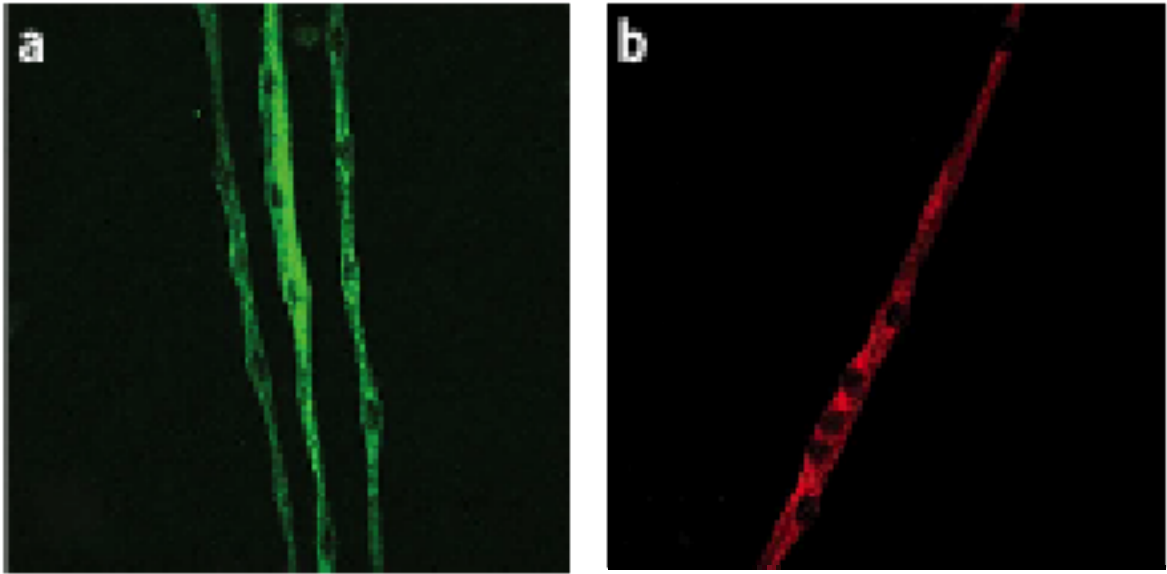


Figure 16. Immunostaining pictures of myotubes. A. Alpha-actin immunostained picture of 7 day old myotubes B. Embryonic myosin heavy chain stained picture of a 7 day old myotube.

In 50% of the cantilevers from each experiment, one will observe that dissociated muscle cells aligned along the long axis of the cantilever to form contracting myotubes (Figure 17).

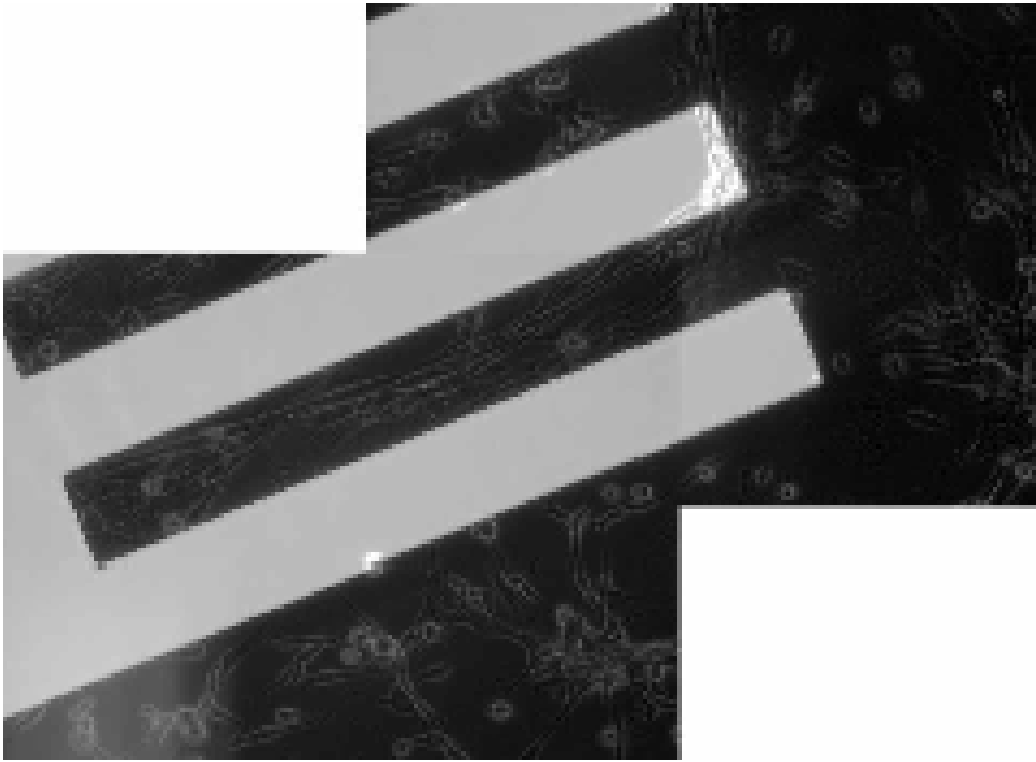


Figure 17. 7 days old pictures of the myotubes growing on the cantilevers. Myocytes are aligning along the long axis of the cantilever and forming contracting myotubes.

Electrophysiological recordings from the myotubes indicate the presence of, voltage-dependent inward and outward currents in voltage clamp mode. In current clamp mode the myotubes fire single action potentials (Figure 18).

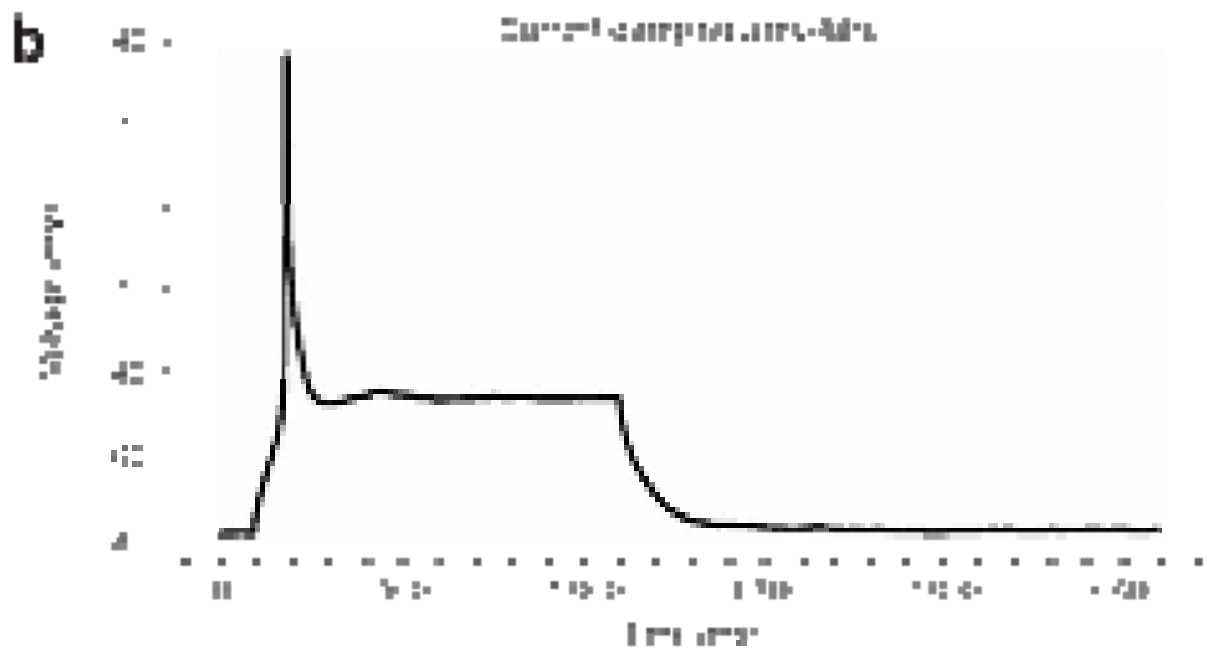
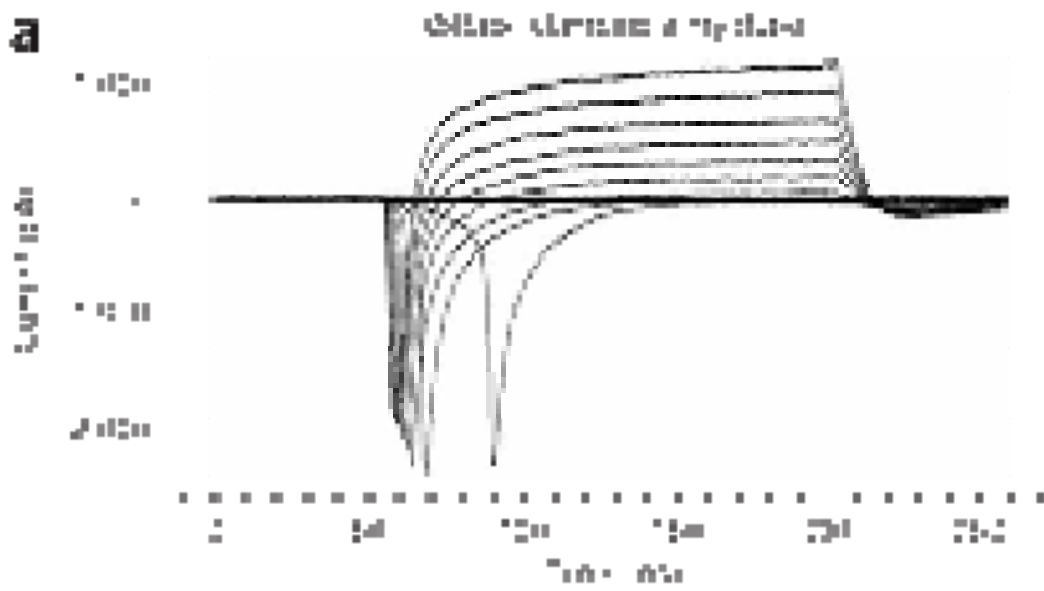


Figure 18. Electrophysiological recordings. Panel A indicates the representative voltage clamp traces obtained after patching a 7 day old myotube. Panel B showing the representative current clamp traces obtained after patching a 7 day old myotube.

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CHAPTER 5: SKELETAL MUSCLE TISSUE ENGINEERING: AN IMPROVED MODEL PROMOTING LONG TERM SURVIVAL OF MYOTUBES, STRUCTURAL DEVELOPMENT OF EXCITATION-CONTRACTION (E-C) COUPLING APPARATUS AND NEONATAL MYOSIN HEAVY CHAIN (NHMC) EXPRESSION

Introduction

Skeletal muscle differentiation and maturation is a complex process that involves the synergy of different growth factors and hormones interacting over a broad time range [1-11]. The differentiation process is further complicated by neuronal innervation, where neuron to muscle cell signaling can regulate myosin heavy chain (MHC) gene expression and acetylcholine receptor clustering [12-18]. Consequently, a basic understanding of how growth factors, hormones and other cell types influence skeletal muscle differentiation will be a key step in the creation of functional tissue engineered constructs, development of advanced strategies for regenerative medicine as well as integrating functional muscle with bio-hybrid MEMS devices for non-invasive interrogation.

One approach in understanding the role of growth factors and hormones in muscle differentiation is to develop an *in vitro* model system that consists of a chemically defined medium supplemented with different growth factors and hormones. This model will offer the opportunity to evaluate the role of each factor. Furthermore, this basic understanding will provide the opportunity to manipulate both components of the medium as well as their temporal application in order to

manipulate the maturation of a slow twitch or fast twitch, extrafusal fibers or intrafusal fiber subtypes.

In vivo, during muscle fiber development, several critical changes occur that indicate functional maturation of the myotubes. These changes include myosin heavy chain (MHC) class switching [19], sarcomere organization [20-23], clustering and colocalization of ryanodine (RyR) and dihydropyridine (DHPR) receptors [24]. A major prerequisite for a successful *in vitro* model for skeletal muscle development needs to satisfy these muscle development requirements mentioned above.

Previously, we had developed a defined system, which promoted differentiation of different skeletal muscle phenotypes and resulted in the formation of contractile myotubes. We showed short-term survival of the myotubes [25, 26]. A novel bio-hybrid technology was developed to integrate the functional myotubes into the cantilever based bio-MEMS device utilized to study muscle physiology, neuromuscular junctions, biorobotics and the stretch reflex arc [27]. More recently, using our defined model system, we achieved a major breakthrough by creating the sensory element of the stretch reflex arc in a petri dish, the intrafusal fibers. The intrafusal fibers are the building blocks of the muscle spindle, which functions as the sensory receptor of the stretch reflex circuit [16]. This was achieved by manipulating the growth factor requirements of the myotubes in our defined system [28].

Although, during the past five years, we have utilized our model system for different applications, it still needs further improvement. The life span of the myotubes in culture needs to be increased, in order to study the development and maturation of slow twitch or fast twitch, extrafusal fibers or intrafusal fiber subtypes, expression of a more mature isoforms of myosin heavy chain proteins in culture and retaining most of the functional characteristics of a mature muscle.

In this paper, we have significantly improved our previous models. This improved model system, with a new modified medium formulation, has the following advantages over the previously published models:

1. This model promotes long-term survival of the myotubes. In this modified culture system the myotubes survived for 50 to 70 days, where in our previous model myotubes survived for 20-24 days.
2. The myotubes exhibited characteristic striations, which has been a common feature with mature skeletal muscle and not observed in our previous model. This lacuna was due to short-time survival of the myotubes in the previous model.
3. In this improved model system, a small fraction of myotubes expressed neonatal myosin heavy chain antibody (N3.36), which is a more mature phenotypic marker for skeletal muscle. In our previous model, we did not observe the expression of these neonatal myosin heavy chain proteins.
4. In this improved model system, most of the myotubes exhibited clustering of ryanodine receptor and dihydropyridine binding complex (Ca^{2+} -Subunit) proteins. These DHPR/RyR clusters

corresponded to the junctional domains of the transverse tubules (T-tubules) and sarcoplasmic reticulum (SR), respectively, and were key in the excitation-contraction (E-C) coupling.

This improved model system, along with the new findings, support our goal of creating functional, tissue engineered, muscle constructs and puts within reach the goal of skeletal muscle grafts. Furthermore, we believe this improved model will be a powerful tool in developing advanced strategies for regenerative medicine in muscular dystrophy and integrating muscle with bio-hybrid prosthetic devices.

Methods and Materials

Surface modification

Glass coverslips (Thomas Scientific 6661F52, 22 x 22mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried [25].

Surface characterization

To characterize the surfaces, contact angle measurements were taken using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1s and C1s scans, utilizing monochromatic Al K α excitation, were obtained [25].

Skeletal muscle culture and serum free medium

The skeletal muscle was dissected from the thighs of the hind limbs of fetal rat (17-18 day-old). The tissue was collected in a sterile 15-ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically

dissociated using 1 ml of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath (100 rpm). After 30 minutes the trypsin solution was removed and 2 ml of L15 + 10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was mechanically triturated and the supernatant was then transferred to a 15 ml centrifuge tube. The process was repeated two times by adding 2 ml of L15 + 10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300 g for 10 minutes at 4°C. The pellet obtained was washed 5 times with L15 medium, then resuspended in 10 ml of L15 and plated in 100 mm uncoated dishes for 30 min. The non-attached cells were removed and centrifuged on a 4% BSA

Figure 19. Schematic diagram showing the culture protocol.

cushion [25]. According to the protocol illustrated in Figure 19, the pellet was resuspended in the serum-free medium and plated on the coverslips. The cells were plated at a density of 700-1000 cells/mm² and attached to the substrate in 1 h. The serum-free medium that contained different growth factors and hormones was added to the culture dish after 1 h. The serum-free medium was prepared by mixing Medium 1 (Table 5) and Medium 2 (Table 6) in a 1:1 v/v ratio. Figure 19 shows the flowchart of the improved culture protocol. Table 5 and Table 6 indicate the growth factor and hormone supplemented compositions of Medium 1 and Medium 2. The cells were maintained in a 5% CO₂ incubator (relative humidity 85%). The full medium was replaced at day 4 with NB4 Activ medium [29] (according to the protocol in Figure 19). Thereafter three-fourths of the medium was changed every three days with NB4 Activ. The protocol was validated in more than fifty trials (n>50).

Table 5: Composition of medium 1.

S. No	Component	Amount	Catalogue #	Source	References
1.	Neurobasal A	500 ml	10888	Gibco/ Invitrogen	[30]
2.	Antibiotic-Antimycotic	5 ml	15240-062	Gibco/ Invitrogen	
3.	Glutamax	5 ml	35050-061	Gibco/ Invitrogen	
4.	B27 Supplement	10 ml	17504-044	Gibco/ Invitrogen	[30, 31]
5.	G5 Supplement (100X)	5 ml	17503-012	Gibco/ Invitrogen	[32-41]
6.	VEGF ₁₆₅ r Human	10 µg	P2654	Gibco/ Invitrogen	[42-45]
7.	Acidic FGF	12.5 µg	13241-013	Gibco/ Invitrogen	[32, 39, 41, 46-51]
8.	Heparin Sulphate	50 µg	D9809	Sigma	[32, 39, 41, 46-51]
9.	LIF	10 µg	L5158	Sigma	[52-60]
10.	Vitronectin (Rat Plasma)	50 µg	V0132	Sigma	[61, 62]
11.	CNTF	20 µg	CRC 401B	Cell Sciences	[63-67]
12.	NT 3	10 µg	CRN 500B	Cell Sciences	[15]
13.	NT 4	10 µg	CRN 501B	Cell Sciences	[68, 69]
14.	GDNF	10 µg	CRG 400B	Cell Sciences	[70-74]
15.	BDNF	10 µg	CRB 600B	Cell Sciences	[69, 75, 76]
16.	CT-1	10 µg	CRC 700B	Cell Sciences	[77-85]

Table 6: Composition of medium 2

S. No	Component(s)	Amount	Catalogue	Source	References
1.	Neurobasal A	500 ml	10888	Invitrogen/Gibco	[30]
2.	Glutamax	5 ml	35050-061	Invitrogen/Gibco	
3.	Antibiotic-antimycotic	5 ml	15240-062	Invitrogen/Gibco	
4.	B27 supplement	10 ml	17504-044	Invitrogen/Gibco	[30, 31]
5.	Cholesterol (250X)	5ml	12531	Invitrogen/Gibco	[86]
6.	TNF-alpha, human	10 µg	T6674	Sigma-Aldrich	[23, 87, 88]
7.	PDGF BB	50 µg	P4056	Sigma-Aldrich	[52, 89-92]
8.	Vasoactive intestinal peptide (VIP)	250 µg	V6130	Sigma-Aldrich	[93]
9.	Insulin-like growth factor 1	25 µg	I2656	Sigma-Aldrich	[58, 59, 88]
10.	NAP	1mg	61170	AnaSpec, Inc.	[94, 95]
11.	Recombinant Apolipoprotein E2	50 µg	P2002	Panvera, Madison, WI	[96]
12.	Laminin, mouse purified	2 mg	08-125	Millipore	[97-103]
13.	Beta amyloid (1-40)	1mg	AG966	Millipore	[104-106]
14.	Human Tenascin-C protein	100 µg	CC065	Millipore	[107]
15.	rr-Sonic hedgehog, Shh N-terminal	50 µg	1314-SH	R&D Systems	[7, 108-118]
16.	rr (Agrin C terminal)	50 µg	550-AG-100	R&D Systems	[119, 120]

Immunocytochemistry of skeletal muscle

Coverslips were prepared for immunocytochemical analysis as previously described. Briefly, coverslips were rinsed with PBS, fixed in -20°C methanol for 5-7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 10 minutes, and blocked for 2h with 10% goat serum and 1% BSA. Cells were incubated overnight with primary antibodies against embryonic myosin heavy chain (F1.652) (dilution $>1:5$), neonatal myosin heavy chain (N3.36) (dilution $>1:5$) (Developmental Studies Hybridoma Bank), ryanodine receptor (AB9078, Millipore) (dilution $>1:500$) and dihydropyridine binding complex (α 1-Subunit) (MAB 4270, Millipore) (dilution $>1:500$) diluted in the blocking solution. Cells were washed with PBS and incubated with the appropriate secondary antibodies for 2 hours in PBS. After 2 hours, the coverslips were rinsed with PBS, mounted on glass slides and observed in a confocal microscope [25, 26, 121].

AChR labeling of the myotubes

AChRs were labeled as previously described [12, 121] by incubating cultures with 5×10^{-8} M of alpha-bungarotoxin, Alexa Fluor® 488 conjugate (B-13422; Invitrogen/Molecular Probes, Carlsbad, CA, USA) for 1.5 h at 37°C before observation. Following incubation in α -bungarotoxin, the cultures were fixed, as mentioned above, for further staining with embryonic myosin heavy chain (F1.652) antibodies.

Patch clamp electrophysiology of the myotubes

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope, as previously describe. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Leibovitz medium, 35°C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, MgCl₂ 2, Na₂ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg, Hepes 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed using a Multiclamp 700A amplifier (Axon, Union City, CA). An Axon Digidata 1322A interface filtered the signals at 2 kHz and digitized at 20 kHz. pClamp 8 software (Axon) recorded and analyzed the data. Membrane potentials were corrected by the subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp 8 program. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -85 mV holding potential. Action potentials were evoked with 1 s depolarizing current injections from a -85 mV holding potential [25, 26].

Results and Discussion

DETA surface modification and characterization

Static contact angle and XPS analysis was used to validate the surface modifications and for monitoring the surface quality. Stable contact angles ($40.64^\circ \pm 2.9$ /mean \pm SD) throughout the study indicated a high reproducibility and quality of the DETA coatings. This was similar to previously published results [25, 26, 31, 121, 122]. Based upon the ratio of the N (401 and 399 eV) and the Si 2p_{3/2} peaks, XPS measurements indicated that a complete monolayer of DETA formed on the coverslips.

Development of serum free medium formulation and the culture technique

The serum-free medium composition was developed empirically. The final medium was derived from two different medium compositions enumerated in Tables 5 and 6. Table 5 constituted the same medium composition that we had previously used for the motoneuron-muscle co-culture and adult spinal cord neurons culture [121, 123-125]. Table 6 constituted twelve additional factors, which had been shown to promote skeletal muscle growth and neuromuscular junction formation. The final medium was prepared by mixing these two mediums in a 1:1 v/v ratio. After the first 4 days of culture, the whole medium was replaced with NB4Activ medium [29]. Thereafter, three-fourth medium was changed after every three days with NB4Activ. Figure 19 shows the culture technique in the flowchart.

Long-term survival and maturation of myotubes

Using our new medium formulation, we were able to successfully culture myotubes for more than 50 days. Figure 20 shows 50-day-old myotubes in culture. As the myotubes aged and grew, they began to form the characteristic anisotropic (A band) and isotropic (I band) banding pattern seen on in vivo muscle fibers [21, 22]. This banding pattern was caused by differential light diffraction due to the organization of myofibril proteins forming sarcomeres within the myotubes [21, 22]. The arrowheads in the images (Figure 20 A-D) indicate myotubes where sarcomeric organization has occurred and was visualized by the appearance of A and I bands.

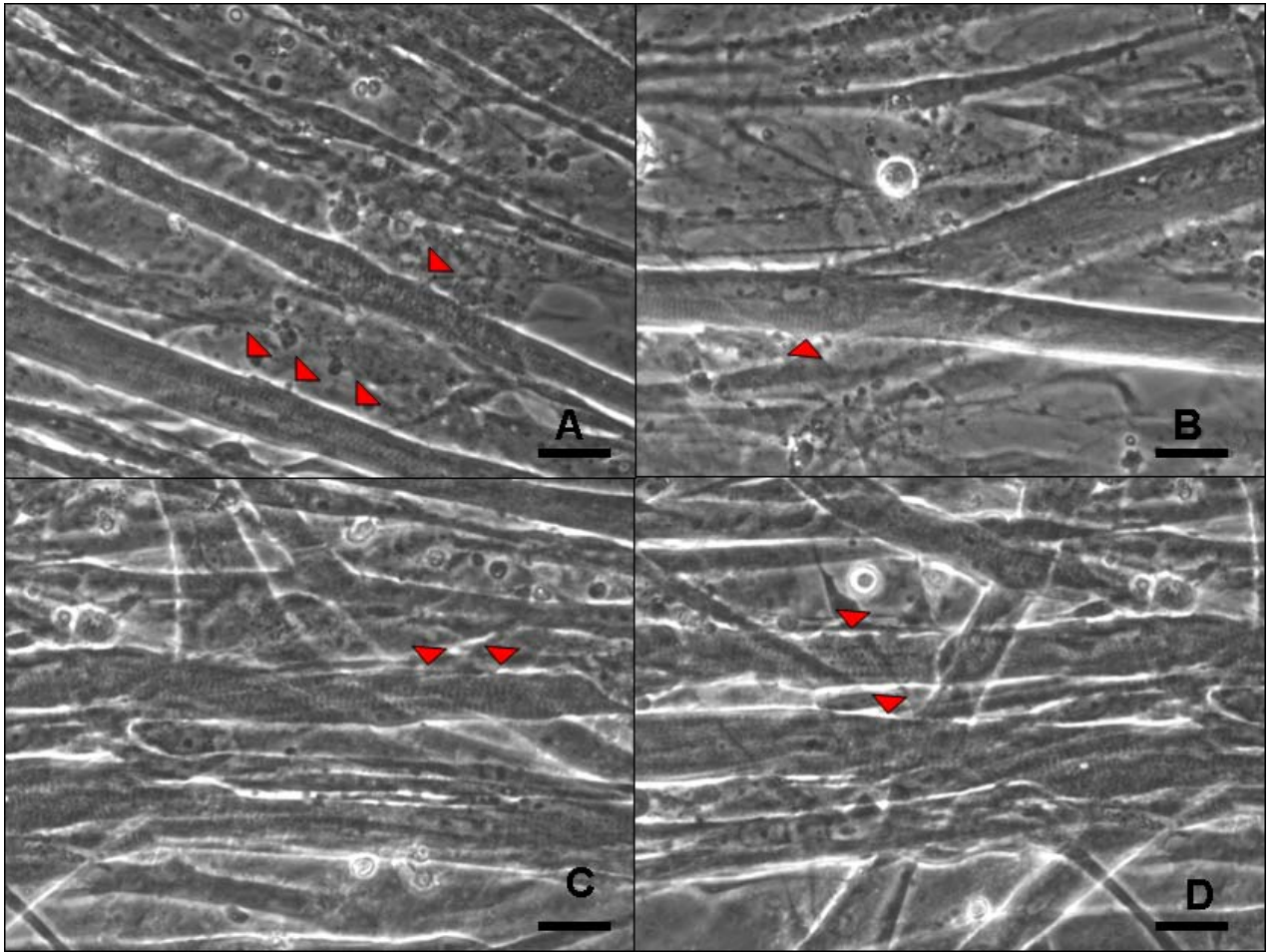


Figure 20. A, B, C and D: Phase pictures of 50-day-old myotubes in culture. Red arrows show characteristic striations in most of the myotubes. Scale bar: 75 micron.

Myotube expression of fetal myosin heavy chain

We evaluated the myotubes formed in this new medium formulation for the expression of fetal MHC. This was to ensure that the new medium formulation was at least mimicking our previous medium [25]. In Figure 21 indicated the characteristic types of myotubes formed at approximately day 50 *in vitro*. These myotubes range from having clustered nuclei (Figure 21 A-D) to having diffuse nuclei organization (Figure 21 E-H). The arrowheads in the images indicate the characteristic striations.

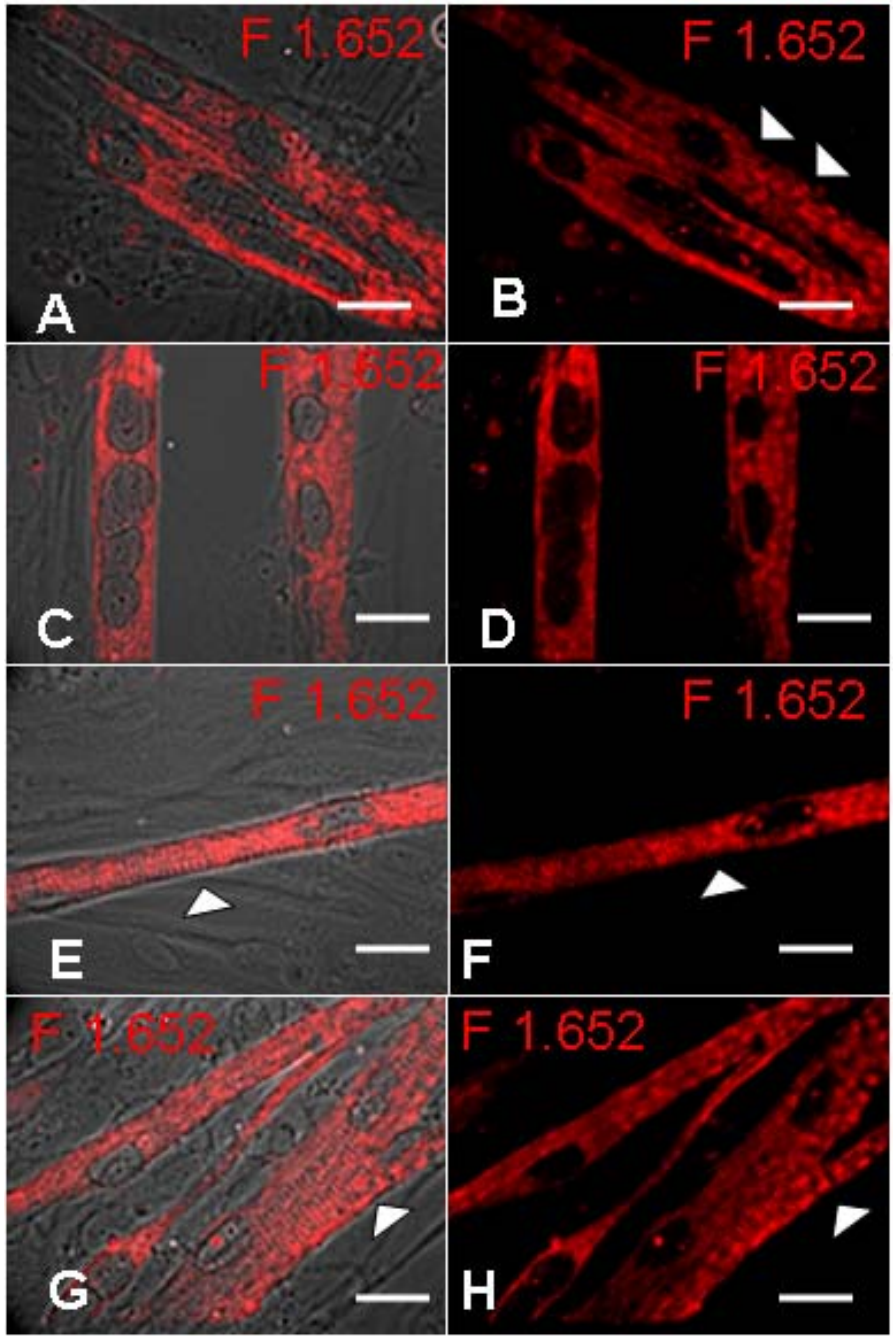
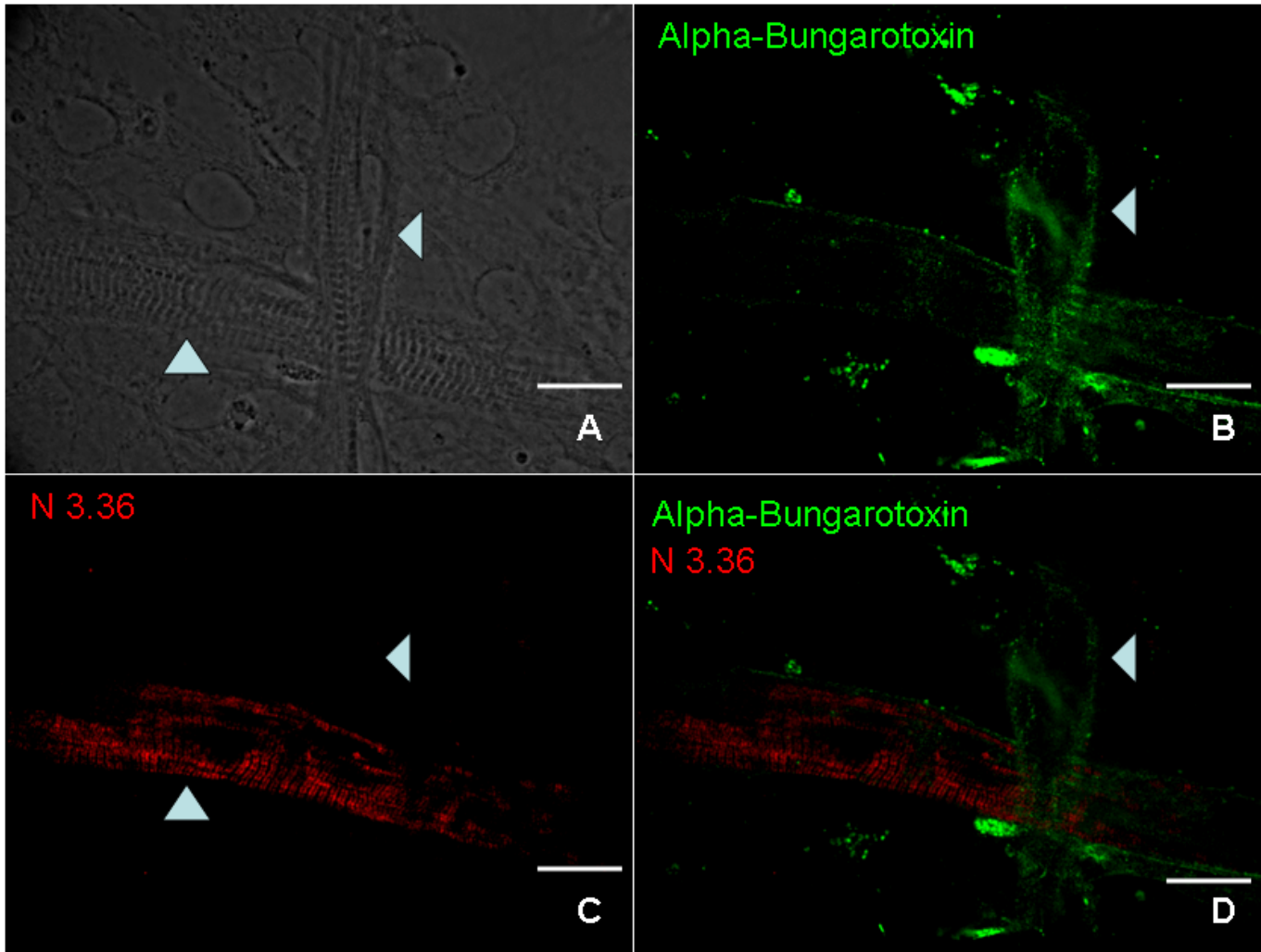


Figure 21. Myotubes stained with antibodies against embryonic myosin heavy chain (F 1.652) proteins at day 50. Scale bar: 75 micron. A. Panel showing phase + fluorescent picture of the myotubes. B. Panel A observed only under fluorescent light. C. Panel showing phase + fluorescent picture of the myotubes. D. Panel C observed only under fluorescent light. E. Panel showing phase + fluorescent picture of the myotubes. F. Panel E observed only under fluorescent light. G. Panel showing phase + fluorescent picture of the myotubes. H. Panel G observed only in fluorescent light. White arrows show the striations in panels B, E, F, G and H.

Differential expression of neonatal myosin heavy chain (N3.36) protein in the myotubes

In order to determine if the myotubes were maturing as they aged *in vitro*, we evaluated the expression of neonatal MHC. After approximately 50 days *in vitro*, 25% of the myotubes began expressing neonatal MHC (Figure 22 A-M). Additionally, we stained the myotubes for clustering of acetylcholine receptors (AChR) using alpha bungarotoxin (Figure 22 B,F). This clustering of AChR receptors are the hot spots where a muscle receives the chemical signals from motoneurons.



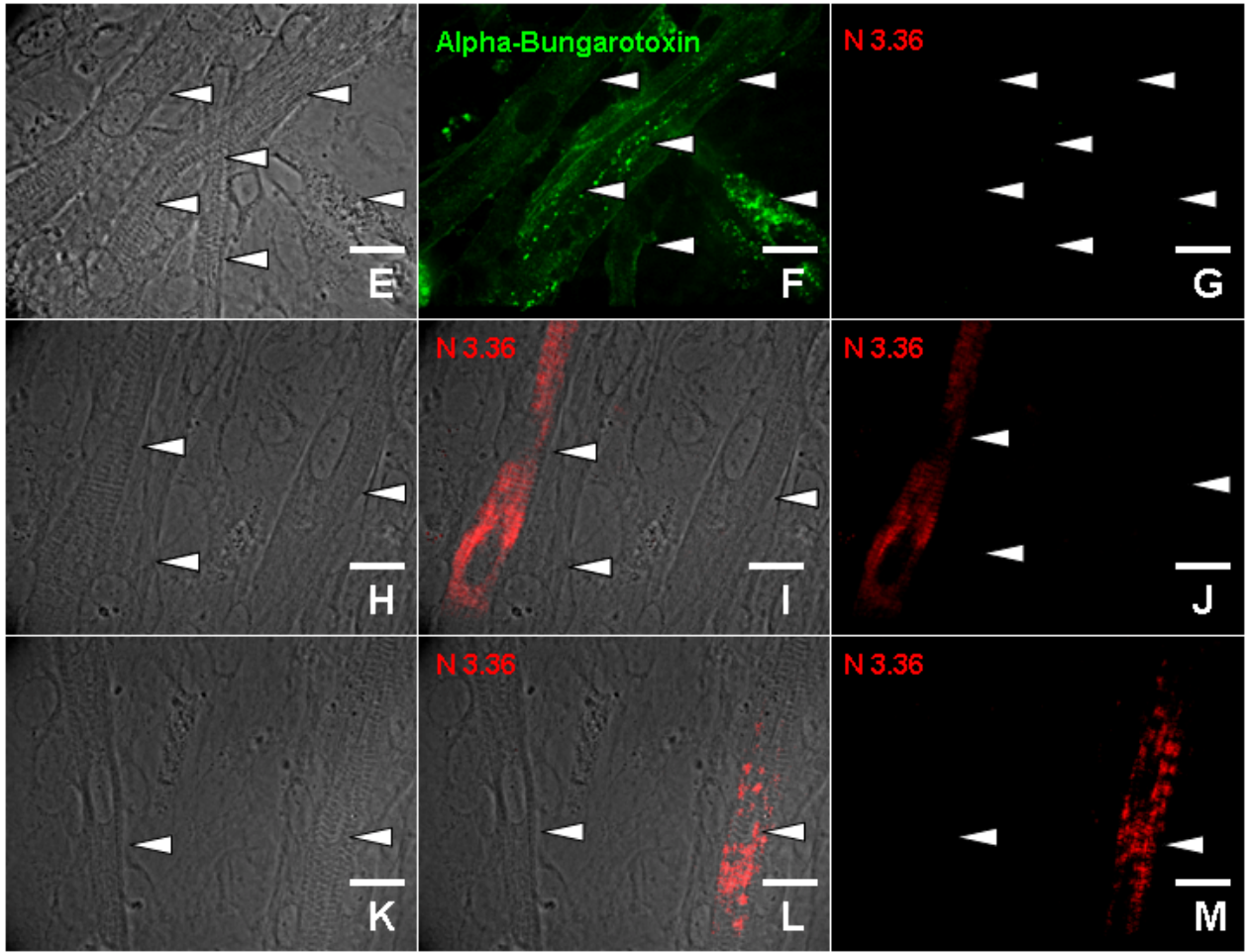
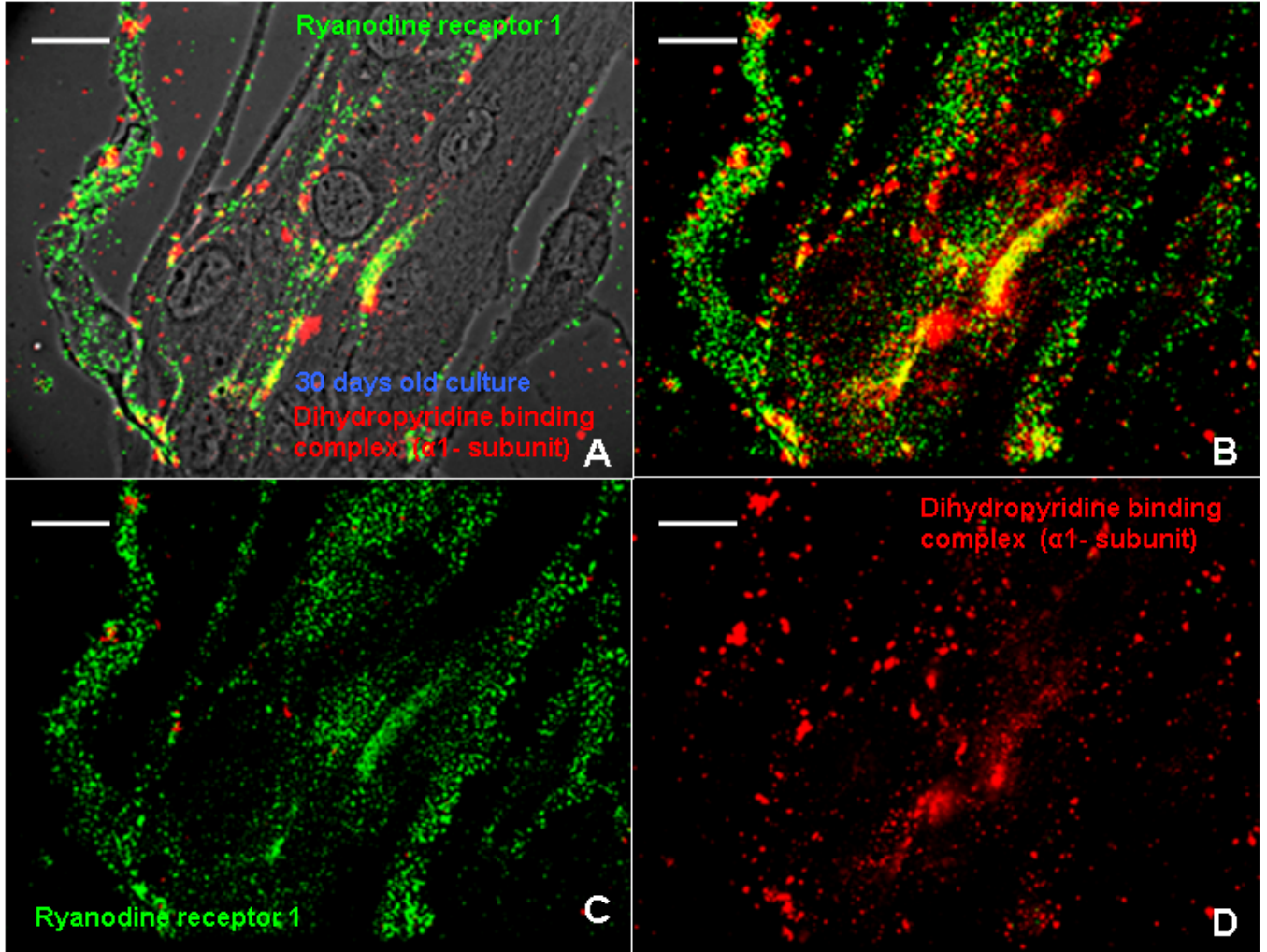
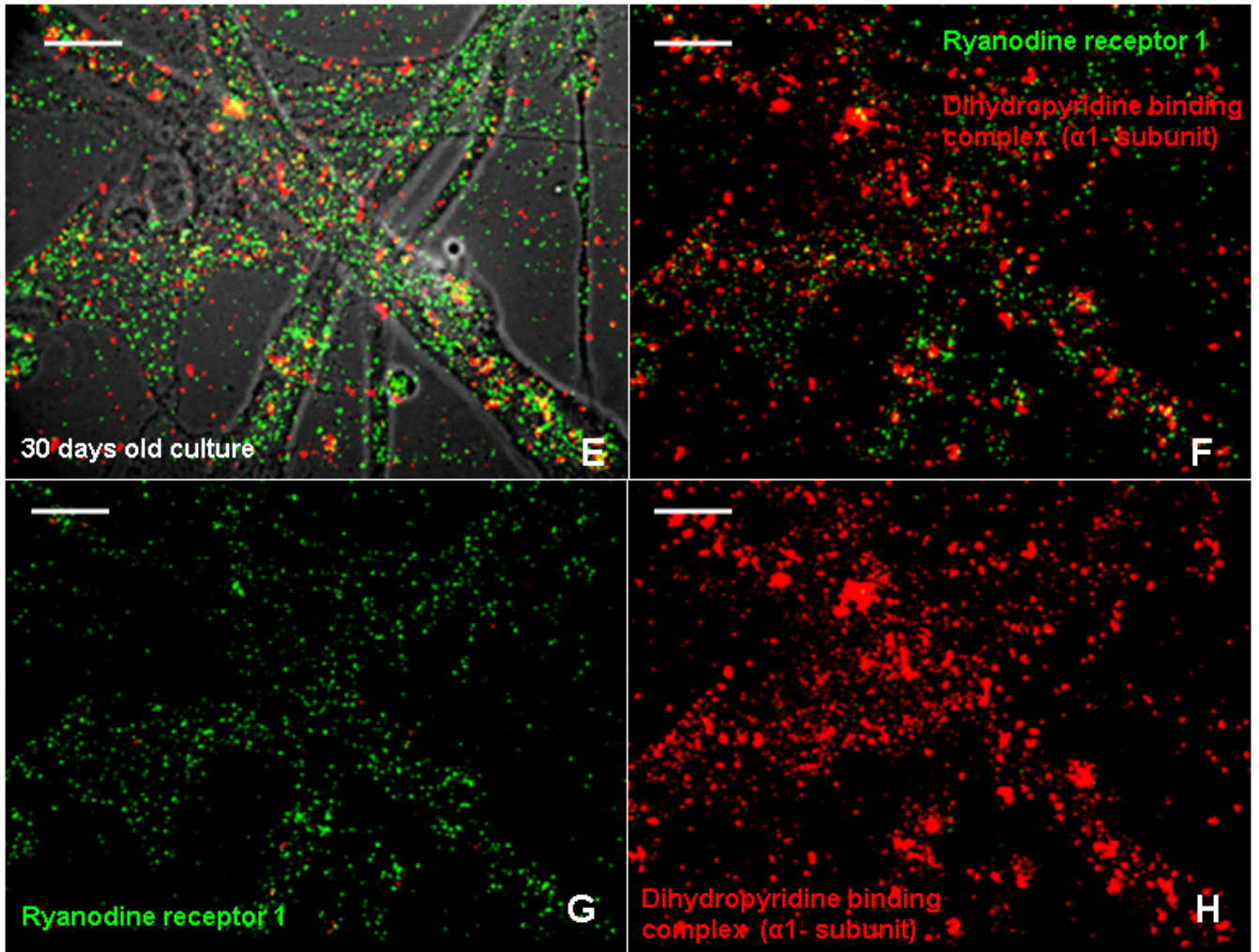


Figure 22. Myotubes immunostained with neonatal myosin heavy chain (N3.36) and alpha-bungarotoxin at day 50. Scale bar: 75 micron. A. Phase picture of 2 myotubes shown by the white arrows. B. Both myotubes shown in phase (Fig A) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining. C. Only one myotube out of the two seen in Fig A stained for N3.36. D. Double stained picture of the Fig A with alpha-bungarotoxin and N3.36. E. Phase picture of 6 myotubes, shown by white arrows. F. All the myotubes shown in phase (Fig E) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining. G. None of the myotubes in Fig E stained for N3.36. H, I, J. Differential staining of the myotubes with N3.36. K, L, M. Differential staining of the myotubes with N3.36.

Ryanodine receptor and dihydropyridine receptor expression and colocalization

The presence of ryanodine (RyR) and dihydropyridine (DHPR) receptor clusters, as well as their colocalization *in vivo*, represents the development of excitation-contraction coupling apparatus in skeletal muscle myotubes [20-22, 24]. We observed the clustering of both RyR and DHPR receptors on the myotubes at 30-day-old in culture (Figure 23 A-D). The clustering and colocalization of RyR + DHPR clusters was observed on different morphologies of the myotubes (Figure 23 E-L). This functional adaptation illustrated that the medium formulation facilitated not only the structural maturation, but also the functional maturation of myotubes in this *in vitro* system.





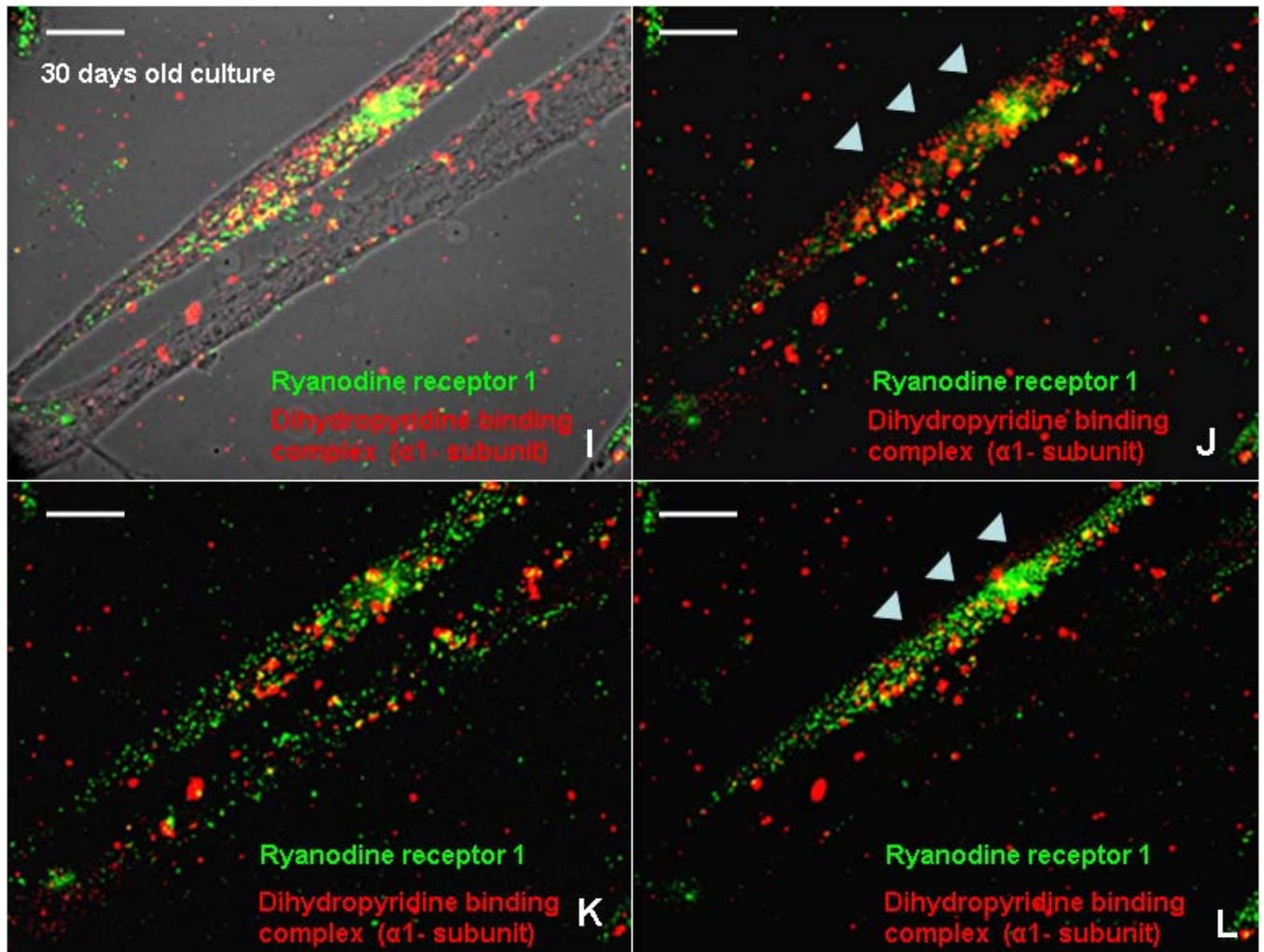
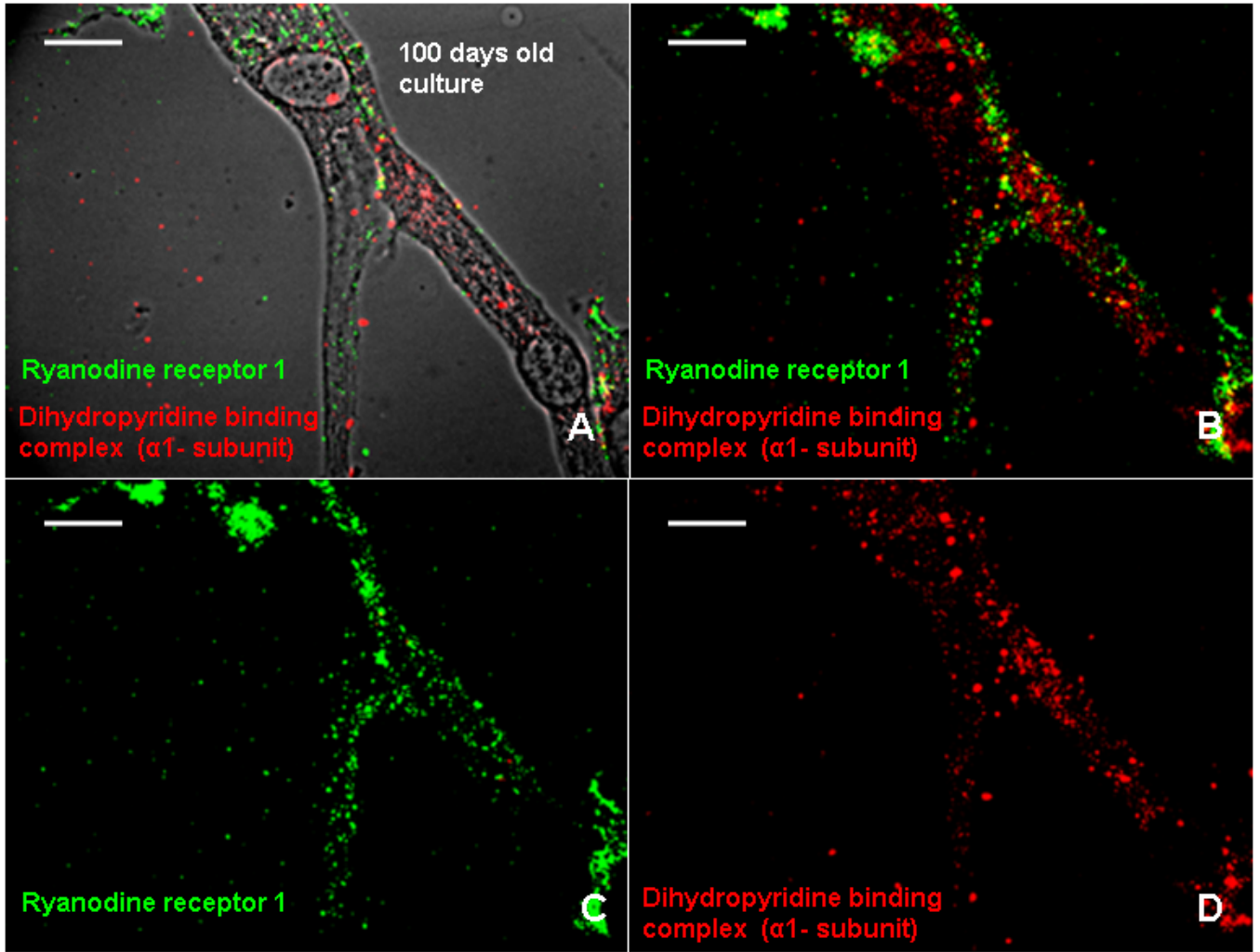


Figure 23. Ryanodine receptor and DHPR receptor clustering in 30-day-old skeletal muscle culture. Scale bar 75 micron. A. Phase and fluorescent-labeled picture of the myotubes. B. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes shown in panel A. C. Ryanodine receptor (Green) on the myotubes shown in panel A. D. DHPR receptors on the myotubes shown in panel A. E. Phase and fluorescent labeled picture of the myotubes. F. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes (Panel E). G. Ryanodine receptor (Green) on the myotubes (Panel E). H. DHPR receptors on the myotubes (Panel E). I. Phase and fluorescent-labeled picture of the myotubes. J, K and L. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red)

clustering on the myotubes (Panel I) on three different planes. White arrows show the striations and the receptor clustering along the striations.

We also observed the clustering of the RyR + DHPR receptors in 100-day-old myotubes, indicating that the older myotubes also maintained their functionality (Figure 24 A-F).



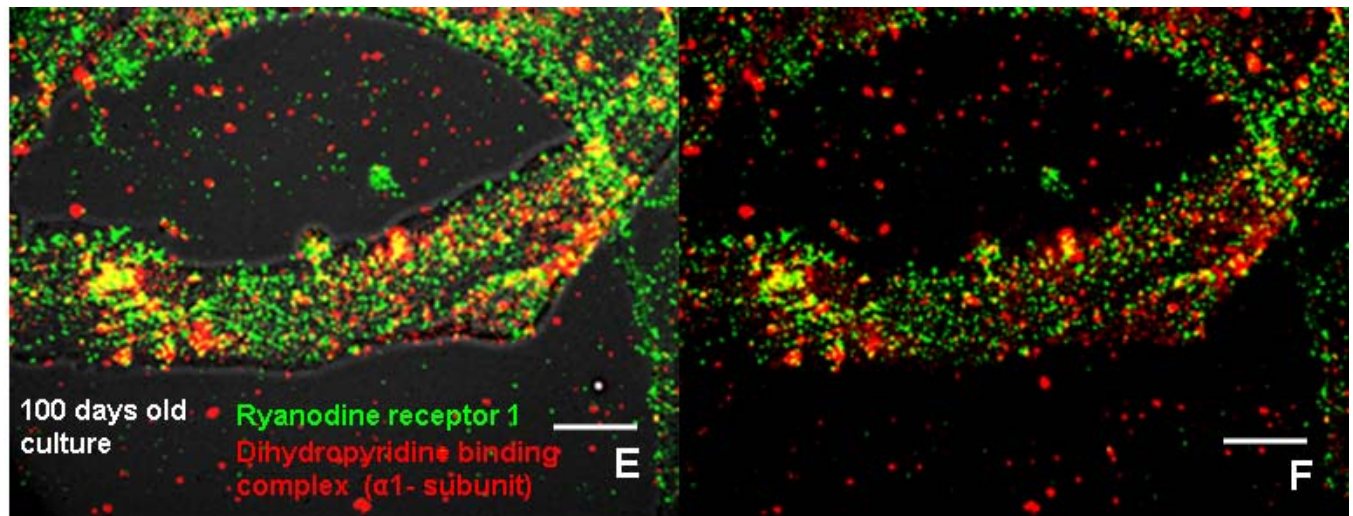
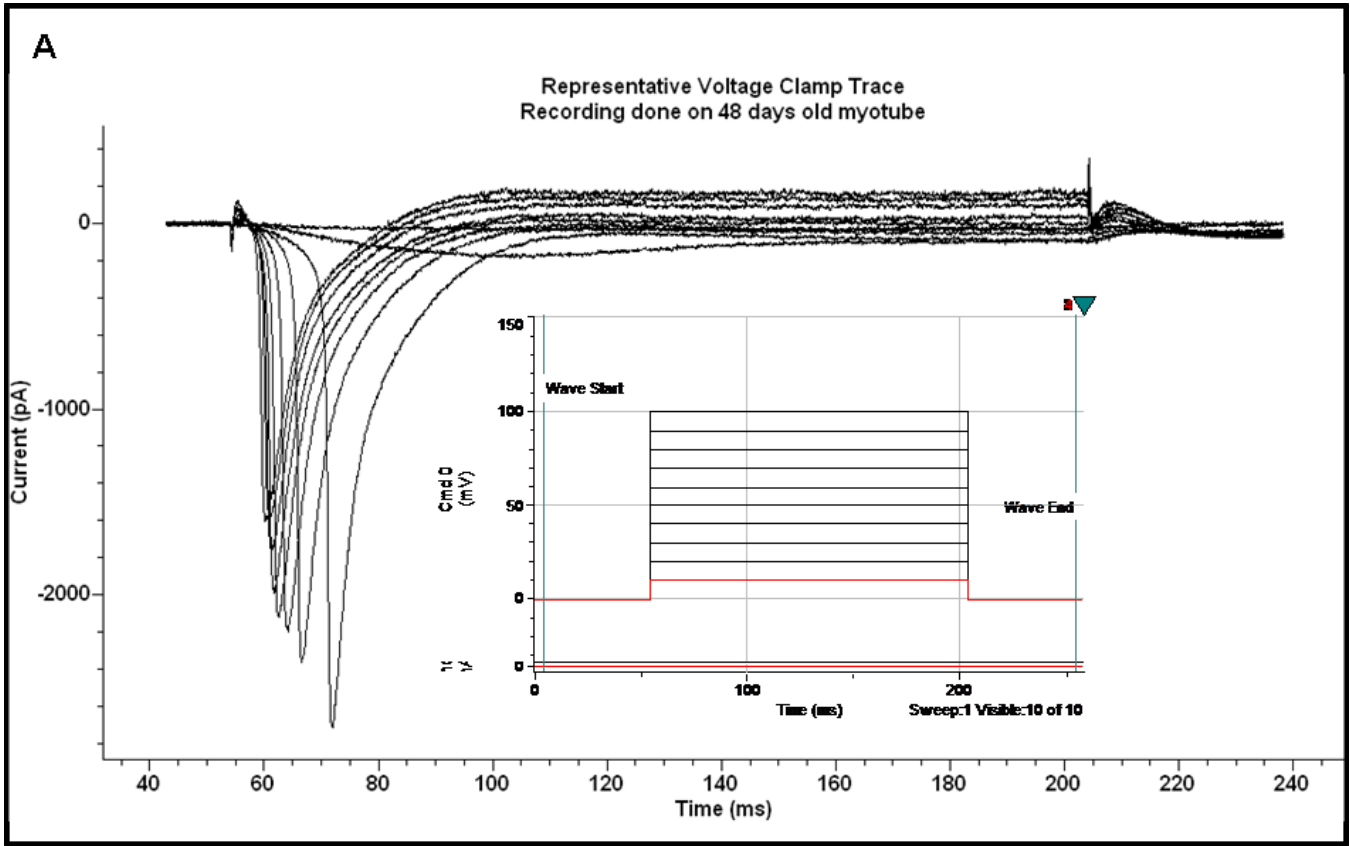


Figure 24. Ryanodine receptor and DHPR receptor clustering in 100-day-old skeletal muscle culture. Scale bar: 75 micron. A. Phase and fluorescent-labeled picture of the myotubes. B. Merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes (Panel A). C. Ryanodine receptor (Green) on the myotubes (Panel A). D. DHPR receptors on the myotubes (Panel A). E and F. Same panels at different plane showing the merged fluorescent picture of the Ryanodine receptor (Green) and DHPR receptor (Red) clustering on the myotubes.

Electrophysiological activity of day 48 myotubes

Myotubes contracted spontaneously in the culture. The contractions were initiated by day 4 in the culture and continued until we maintained the culture. Most of the myotubes expressed functional voltage gated sodium, potassium and calcium ion channels as previously reported [25]. The voltage clamp electrophysiology of the myotubes showed both inward and outward currents, indicating functional sodium and potassium channels (Figure 25 A). The current clamp study showed the single action potential fired by the myotubes (Figure 25 B).



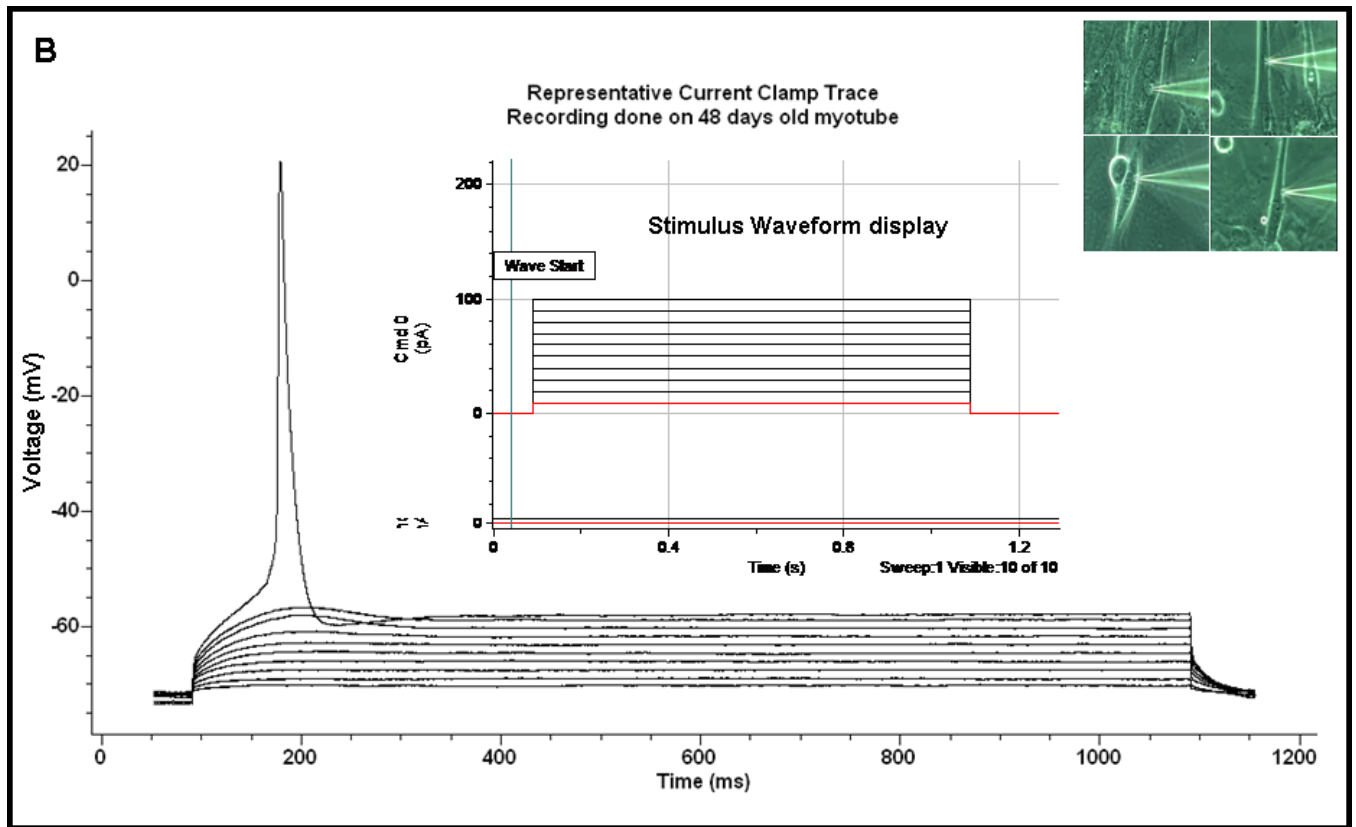


Figure 25. Patch clamp electrophysiology of the myotubes. A. Representative voltage clamp trace obtained after patching a 48-day-old myotube in culture ($n > 20$) B. Representative current clamp trace of the same myotube, which a voltage clamp trace had been obtained ($n > 20$). Inset showing the picture of patched myotubes. (n = number of myotubes which were patched during the course of study)

Our previous studies have shown the biocompatibility of the DETA silane substrate. One interesting feature about DETA silane is that, it partially mimics the three dimensional features of the extra-cellular matrix, which may be partly responsible for the robust growth of the different cell types on a synthetic substrate [25, 26, 31, 121-125].

We have empirically developed a serum-free medium that supports the survival, proliferation and fusion of fetal rat myoblasts into contractile myotubes. The rationale for selecting these growth factors was solely based on the distribution of their receptors within the developing myotubes of a rat fetus [1-11]. In Tables 5 and 6, we have referenced the literature where these individual growth factors, hormones and neurotransmitters had been observed to support muscle and neuromuscular junction development. The composition of Table 5 is same as what we had previously used for the motoneuron-muscle co-culture and adult spinal cord neuron culture [121, 123-125]. In Table 6, we have added twelve more factors, which had been indicated in muscle and neuromuscular junction formation. The other key change in our culture protocol is that we had used NB4Activ [29] for maintenance of the culture. NB4Activ is a specialized medium used for fetal hippocampal neuron growth in culture [29]. We observed that this medium supported the survival of skeletal muscle.

In our previous model, we observed embryonic myosin heavy chain (EMHC) expression. In this study, apart from EMHC, we observed that 25% of the myotubes expressed neonatal myosin heavy chain (NMHC) proteins. NMHC is a more mature marker of the muscle [19]. We believe that

the myotubes were more mature in this culture due to two reasons: 1), the long-term survival was due to a modified protocol and advance medium formulation, and 2) the additional growth factors promoted more mature myotube formation.

The expression of co-localized RyR and DHPR clusters in the myotubes at approximately 50 days and 100 days in culture showed that this modified medium formulation supports the functional maturation of the fibers and formation of excitation-contraction coupling apparatus. Excitation-contraction (E-C) coupling is the signaling process in muscle that membrane depolarization leads to force production [126]. E-C coupling in striated, skeletal muscle is a fast process in which a brief depolarization causes an immediate elevation of the cytosolic Ca^{2+} [127]. This process is brought about by a close interaction between the dihydropyridine receptor (DHPR), L-type Ca^{2+} channel and the ryanodine receptor type 1 (RyR1) present in the skeletal muscle. Close proximity between the DHPR and RyR complexes occurs at specialized junctions established between the transverse tubular and sarcoplasmic reticulum (SR) membranes [128]. At these junctions, voltage-dependent movements of the electrical charges in the skeletal DHPR are coupled to the opening of the RyR1 channel [129-131]. This further leads to the force generation in a striated muscle. This structural adaptation represents a significant functional change due to the fact that excitation-contraction coupling is required for successful muscle development as well as neuromuscular junction formation [20-22, 24]. Therefore, a better understanding of E-C coupling is extremely important in regenerative medicine. In the past for a better understanding of the molecular mechanism of E-C

coupling, researchers had relied heavily on myotube models. But, there was no defined, serum-free model in order to study this supramolecular assembly of E-C coupling apparatus. This improved model offers the potential to study E-C coupling in a defined system. Patch-clamp electrophysiology provided further evidence that the muscle maintained their functional, voltage gated ion channel in the system.

This improved model system along, with these new findings, support our goal for creating functional, tissue engineered, muscle constructs and puts within reach the goal of skeletal muscle grafts. Furthermore, we believe this improved model will be a powerful tool in developing advance strategies for regenerative medicine in muscular dystrophy and integrating muscle with bio-hybrid prosthetic devices.

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Part 2: Tissue Engineering Neuro-Muscular Junction (NMJ)

CHAPTER 6: EMBRYONIC MOTONEURON-SKELETAL MUSCLE CO-CULTURE IN A
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Introduction

In vitro co-culture of mammalian nerve and muscle cells is a popular model system to study neuromuscular junction (NMJ) formation, function and maintenance, as well as nerve-muscle disorders, signal computation at the nerve-muscle interface and a plethora of other applications ranging from biorobotics and tissue engineering to drug screening [1-4]. Currently, all the known *in vitro* nerve-muscle co-culture models use serum containing media [1, 3-6]; this provides a functional model system, but the use of serum adds unwanted variability. Thus, the use of serum makes it impossible to describe or quantify the minimum factors needed to recreate the mammalian NMJ *in vitro*. In this work, we describe the development of a defined motoneuron and muscle co-culture system resulting in the formation of NMJs including: 1) a new culture technique, 2) a novel serum-free medium formulation and 3) a synthetic SAM substrate (N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine - DETA). We characterized the culture by morphology, immunocytochemistry, electrophysiology and videography. This model system provides a better understanding of the necessary growth factor and substrate interactions in NMJ formation and provides an enhanced system for nerve-muscle tissue engineering, regenerative medicine and development of limb prosthetics.

Methods and Materials

Surface modification

Glass coverslips (Thomas Scientific 6661F52, 22×22 mm No. 1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 min at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) film was formed by the reaction of the cleaned surface with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by XPS (Kratos Axis 165) by monitoring the N 1s peak [7-13].

Rat embryonic motoneuron isolation.

Rat spinal motoneurons were purified from ventral cords of embryonic day 14 (E14) embryos. Briefly, rats were anaesthetized and killed by inhalation of an excess of CO₂. This procedure was in agreement with the Animal Research Council of Clemson University and the University of Central Florida, which adheres to IACUC policies. Ventral spinal cells from the embryo were collected in cold Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27. The cells were dissociated with 0.05% trypsin-EDTA (Invitrogen) treatment. The dissociated cells were layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate E/ GlutaMAX™/ antibiotic-antimycotic/

B27) followed by centrifugation for 15 min, using 800g, at 4°C. This is modified from the previously described protocols due to non-availability of metrizamide [10, 12, 14]. After centrifugation, four bands of cells were obtained. The motoneurons constituted the uppermost band with large somas. These cells were further purified by immunopanning. The motoneurons were selected using the immune interaction between the motoneurons and MAB192 antibody (1:2 dilution, ICN Biomedicals, Akron, OH) coated on the dishes[12, 15, 16] . The antibody recognized the low affinity NGF receptor that is only expressed by ventral motoneurons at this age [17]. Purified motoneurons were plated on top of muscle cells at a density of 50 cells/mm².

Muscle cell isolation and culture

The skeletal muscle was dissected from the thighs of the rat fetus (E17) hind limbs. The tissue was collected in a sterile 15 ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 2 ml of 0.05% of trypsin-EDTA solution for 60 min in a 37 °C water bath (100 rpm). After 30 min the trypsin solution was removed and 4 ml of Hib. E +10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated and the supernatant transferred to a 15 ml centrifuge tube. The same process was repeated two times by adding 2 ml of Hib. E +10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in Hib. E medium) cushion and centrifuged at 300g for 10 min at 4 °C. Finally, the cell pellet was suspended in 1 ml of serum-free

medium and plated on 100 mm uncoated dishes for 30 min. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and plated at a density of 700–1000 cells/mm² on DETA coated coverslips. The cells attached to the substrate in 1 h [11].

Plating the embryonic motoneuron on top of muscle

The immuno-pure embryonic motoneurons were plated at a density of 50 cells/mm² on the top of the muscle layer. The serum-free medium was added to the culture dish after 1 h and the cells were maintained at 37°C in a 5% CO₂ incubator (relative humidity 85%) (Figure 26).

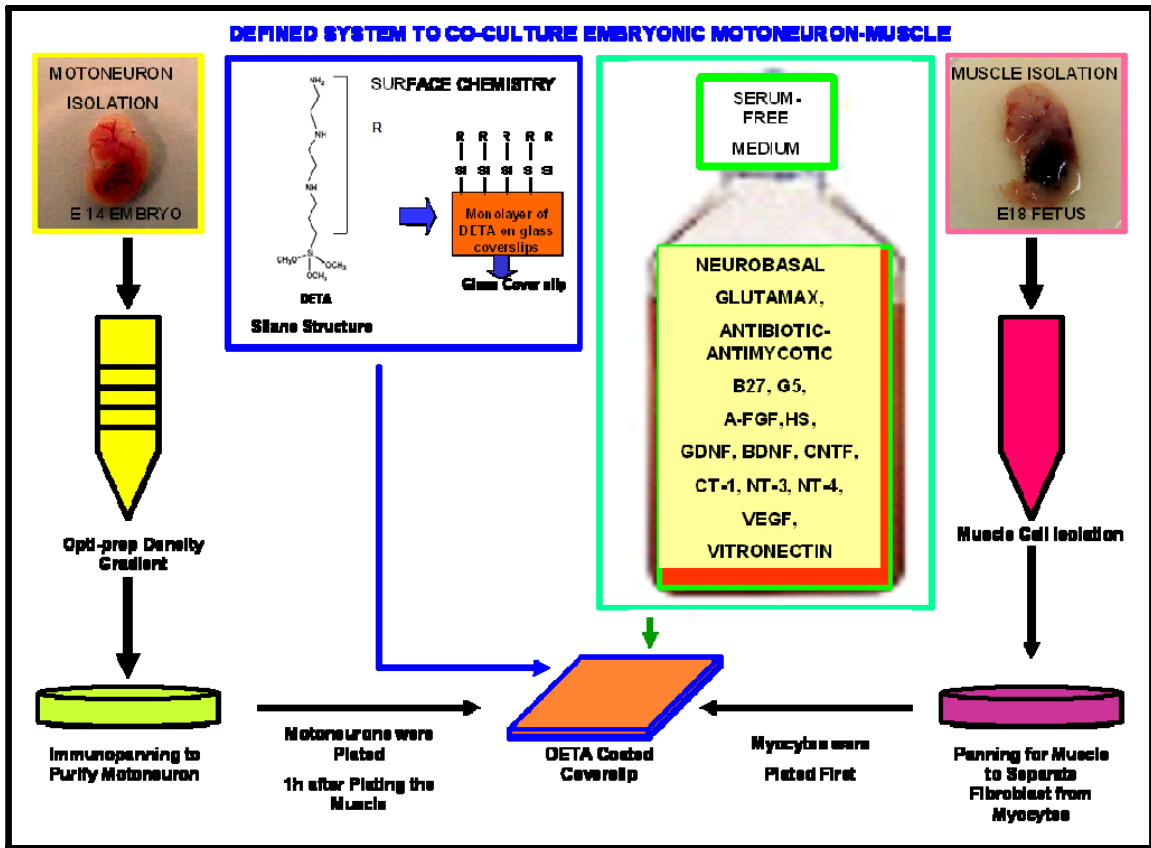


Figure 26. Defined model system for the embryonic motoneuron-muscle co-culture.

Immunocytochemistry

Embryonic myosin heavy chain

Coverslips were rinsed with PBS, fixed in -20°C methanol for 5–7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution), and blocked for 30 min in a permeabilization solution + 10% goat serum (blocking solution). Cells were incubated overnight with primary antibody against fetal MHC (F1.652, IgG, Developmental Studies Hybridoma Bank) diluted (1:5) in the blocking solution. Cells were washed with PBS and incubated with the secondary antibody (diluted in PBS) for 2 h. The secondary antibody solution was removed and the cells were rinsed using PBS. The coverslips were dried and mounted on glass slides using VectaShield+DAPI mounting medium (Vector Laboratories H-1200) and viewed on a confocal microscope (UltraVIEW™ LCI, PerkinElmer)[11, 18].

Double staining with Neurofilament 150 and Embryonic myosin heavy chain

Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4°C with rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:2000) and fetal MHC (F1.652, IgG, Developmental Studies Hybridoma Bank, diluted 1:5). After overnight incubation, the coverslips were rinsed three times with PBS and then incubated with the appropriate secondary antibodies for 2h. After rinsing three times in PBS, the coverslips were mounted with Vectashield+DAPI mounting medium onto glass

slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative.

AChR labeling and observation of co-cultures.

AChRs were labeled as described previously [5] by incubating cultures with 5×10^{-8} M α -bungarotoxin, Alexa Fluor® 488 conjugate (Molecular Probes, B-13422) for 1.5 hr at 37°C before observation. Labeled cultures were fixed with glacial acetic acid and ethanol, washed with PBS, dried, mounted and examined by confocal microscopy.

Colabeling AChRs with α -bungarotoxin and synaptophysin.

AChRs were labeled as described above. The AChRs labeled coculture coverslips were also processed for immunocytochemistry as described above. Cells were incubated overnight with the primary antibody against major synaptic vesicle protein p38, anti-synaptophysin (Chemicon, MAB368, diluted 1:1000) in the blocking solution. Cells were washed with PBS and incubated with the secondary antibody (diluted in PBS) for 2 h. The secondary antibody solution was removed and the cells were rinsed using PBS. The coverslips were dried and mounted on glass slides using VectaShield+DAPI mounting medium (Vector Laboratories H-1200) and viewed on a confocal microscope (UltraVIEW™ LCI, PerkinElmer).

Staining for glial cells and fibroblasts

Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4°C with mouse anti-GFAP, (Chemicon, MAB360, diluted 1:1000) and mouse anti-fibroblasts antibody, clone TE-7 (Chemicon, CBL271, diluted 1:100). After overnight incubation, the coverslips were rinsed three times with PBS and then incubated with the appropriate secondary antibodies for 2h. After rinsing three times in PBS, the coverslips were mounted with Vectashield+DAPI mounting medium onto glass slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative.

Electrophysiology

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Leibovitz medium, 35 °C). The patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, MgCl₂ 2, Na₂ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg, Hepes 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were performed with pClamp 8 software (Axon). Membrane potentials were corrected by the subtraction of a 15 mV tip

potential, which was calculated using Axon's pClamp 8 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from -85 to -95 mV without any whole-cell or series resistance compensation. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -85 mV holding potential. Action potentials were evoked with 1 s depolarizing current injections from a -85 mV holding potential [10-13].

Video recordings

Determination of the effect of (+) – tubocurarine chloride pentahydrate (d-tubocurarine or Curare) on the NMJ by video recording: $5 \mu\text{M}$ of the nicotinic cholinergic antagonist, (+) – tubocurarine chloride pentahydrate (Cat no. 93750, Sigma), was applied in the bath solution in order to block the acetylcholine receptors present in the NMJ.

Results and Discussion

We used embryonic motoneurons and skeletal muscle cells to initiate a developmentally relevant system for the study of NMJ formation. The motoneurons were obtained from the spinal cord of embryonic, day 14, rat embryos. The detailed protocol for motoneuron isolation [12, 15, 16, 19] and muscle isolation [11] has been discussed and reported previously. Briefly, purified motoneurons were isolated in two steps. First, ventral horn motoneurons were separated on an opti-prep density gradient [12, 14]. Then, they were further purified by immunopanning [12, 15]. These purified motoneurons were co-cultured with dissociated skeletal muscle obtained from the hind limb of the day 18 rat fetuses [11]. Current co-culture models call for plating of motoneurons only after myotube formation, adding time and complexity to the system [1, 20, 21]. In the current system, dissociated myocytes were plated and after one hour the motoneurons were added on top of the adherent myocytes. This facilitated the development of both cell types concurrently, yielding a robust culture (Figure 26).

In order to understand the fundamental soluble factors required for NMJ formation, a serum-free, chemically defined media was developed by systematically integrating components from our independently developed culture media for spinal cord motoneurons [10, 12], skeletal muscle [11] and cardiac muscle [13]. Individually, these media provide for the culture of adult and embryonic motoneurons and embryonic myocytes on a synthetic substrate (DETA - a self-assembled monolayer or SAM). We combined certain different factors from these defined media systems and

showed that it provides for the co-culture of embryonic motoneuron-muscle cell types. The cultures were initiated in the defined medium (Table 7). Half of the medium was changed every 4-5 days with Neurobasal+B27+Glutamax+antibiotic/antimycotic and the system provided a maximum survival of 3 weeks.

Table 7: Serum-free co-culture medium composition

Component	Amount/concentration	Company	Catalog number
Neurobasal	500 ml	Invitrogen	10888
B27	10 ml	Invitrogen	17504-044
G5 (100x)	1 ml	Invitrogen	17503-012
aFGF	10 µg	Invitrogen	13241-013
VEGF 165	10 µg	Invitrogen	P2654
Human BDNF	10 µg	Cell Sciences	CRB 600B
Human GDNF	10 µg	Cell Sciences	CRG 400B
Rat CNTF	25 µg	Cell Sciences	CRC 401B
Human CT-1	10 µg	Cell Sciences	CRC 700B
NT-3	10 µg	Cell Sciences	CRN 500B
NT-4	10 µg	Cell Sciences	CRN 501B
Human LIF	10 µg	Sigma	L5283
D- N-acetylated heparin sulfate	40 µg	Sigma	D9809
Vitronectin	50 µg	Sigma	V0132
Glutamax (100x)	5 ml	Invitrogen	35050-061
Antibiotic-antimycotic 100x	5 ml	Invitrogen	15240-062

This system uses a patternable [8, 22], non-biological, cell growth promoting, organosilane substrate (DETA) [7, 9-13, 23, 24], coated on a glass surface, facilitating variable control and reproducibility of the growth surface. Quality of the surface modified coverslips used for the cell culture was evaluated using static contact angle measurements and X-ray photoelectron spectroscopy (XPS) analysis as previously described [9-13, 24, 25]. Stable contact angles ($40.64^\circ \pm 2.9/\text{mean} \pm \text{SD}$) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results [9-13, 24]. Based on the ratio of the N (401 and 399 eV) and the Si $2p_{3/2}$ peaks, XPS measurements indicated that a complete monolayer of DETA was formed on the coverslips (Figure. 26 Blue Box).

Using immunocytochemistry, myotubes were stained using fetal myosin heavy chain antibodies [18] (Figure 27 A, B) at day 12. The immunopure motoneurons were visualized using an antibody to the cytoskeletal protein neurofilament-150 kDa. (Figure 27 C, D).

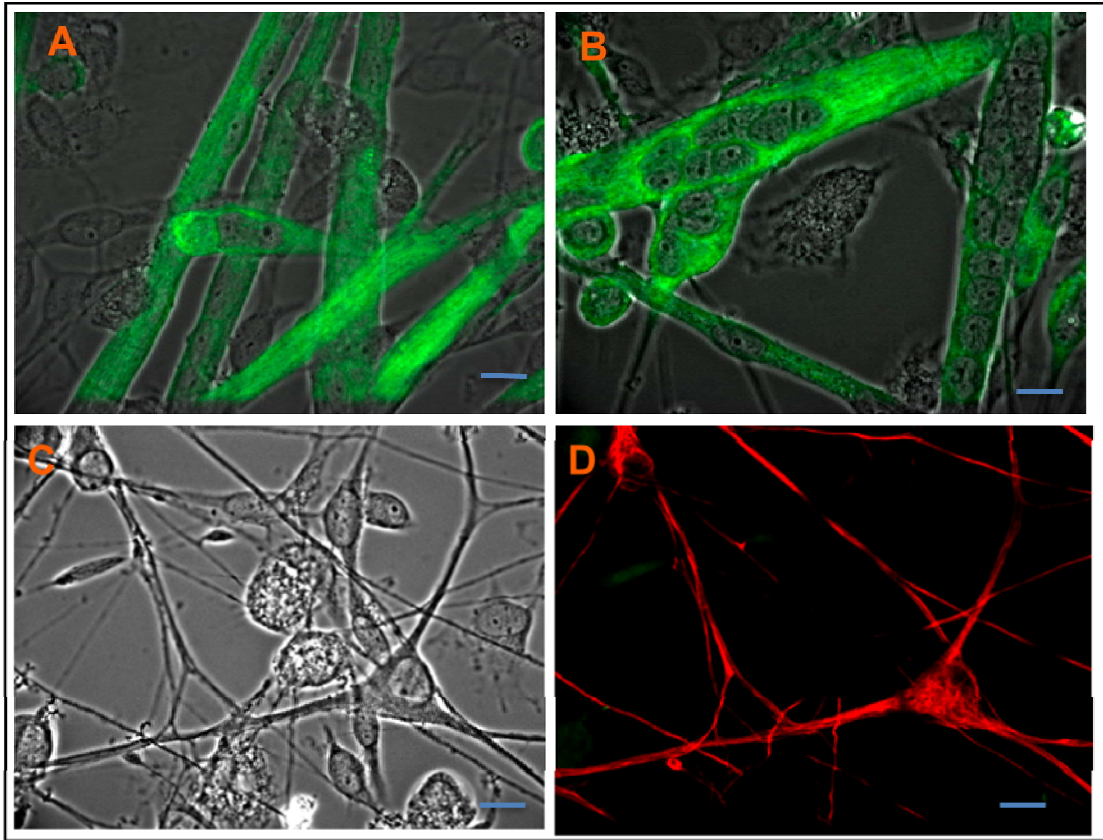


Figure 27. Immunostaining of the myotubes and the motoneurons separately in co-culture. A,B. Myotube morphology and immunocytochemistry in co-culture was done using the fetal myosin heavy chain antibody and appear green. Myotubes and characteristic nuclei can be easily seen. C, Motoneuron morphology in co-culture was visualized using phase contrast microscopy and the cell soma and processes are easily distinguished. D, Motoneuron immunocytochemistry using the NF-150 antibody. Cell soma and processes appear red. Scale bars: 20 microns in all panels.

Co-cultures were visualized by double staining the cultures with fetal myosin heavy chain and neurofilament-150 antibodies (Figure 28 A, B, C, D). The postsynaptic acetylcholine receptor was verified by alpha-bungarotoxin staining of the acetylcholine receptor clustering at the nerve-muscle synapse [5, 26] (Figure 28 E, F).

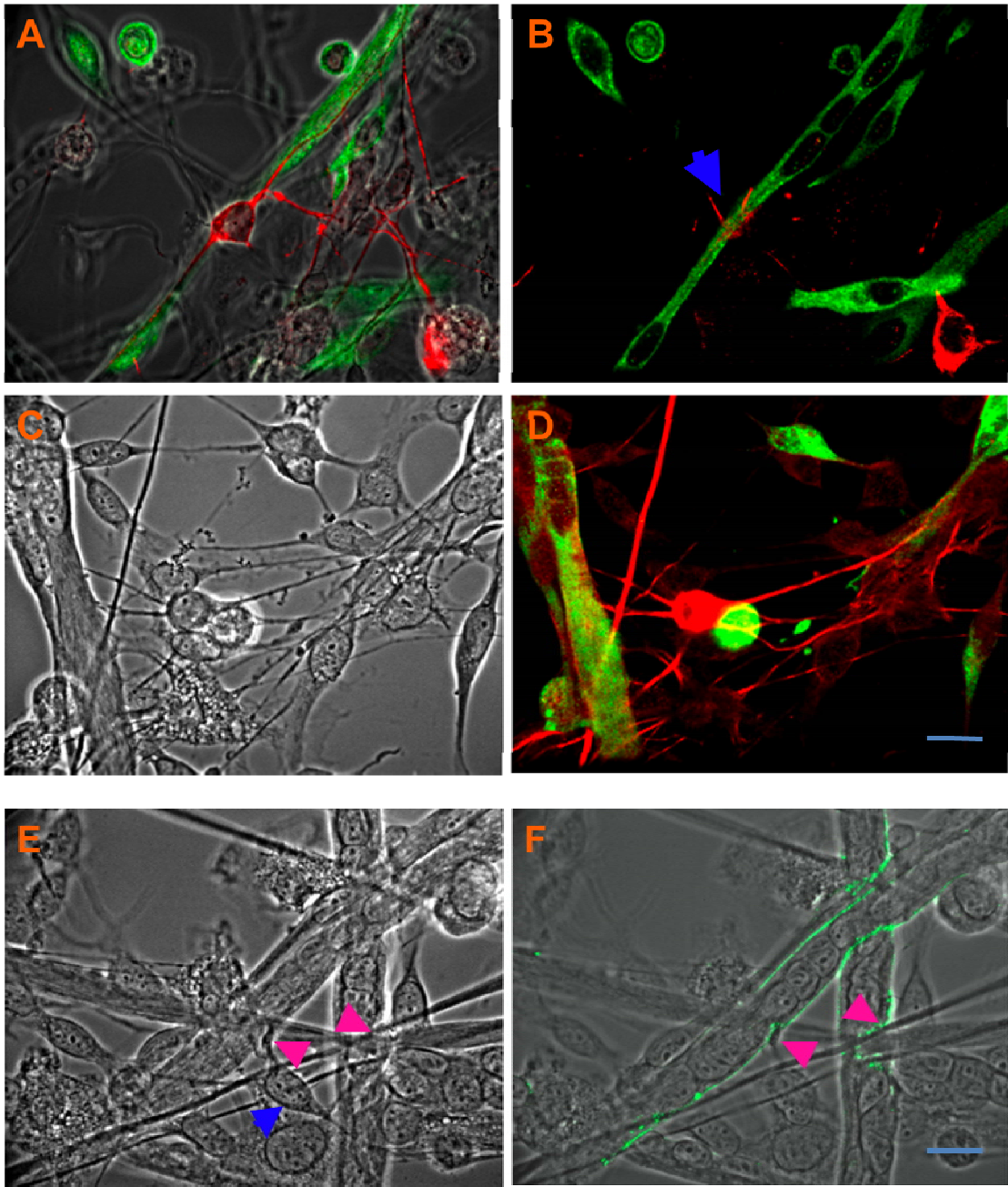


Figure 28. Phase contrast and immunocytochemical characterization of myotubes and motoneurons in co-culture. A,B, Myotubes stained for fetal myosin heavy chain (green) and motoneurons stained for neurofilament 150 (red) are shown in proximity to each other with neuronal processes touching the myotubes in two planes (blue arrows). C,D, Phase contrast and confocal images of a myotube (green) innervated by multiple neuronal processes (red) and in proximity to a motoneuron (red). E,F, phase contrast and alpha-bungarotoxin staining of motoneuron-myotube co-culture, where the blue arrow points to the motoneuron, and red arrows and green dot clusters indicate areas of acetylcholine receptor clustering.

Immunocytochemical evidence for synapse formation between the embryonic motoneuron and myotube at the NMJ was shown by close proximity of the presynaptic marker, synaptophysin (synaptic vesicle protein) and the clustering of the acetylcholine receptors (eg. by alpha-bungarotoxin labeling) in Figure 29 A, B, C, D.

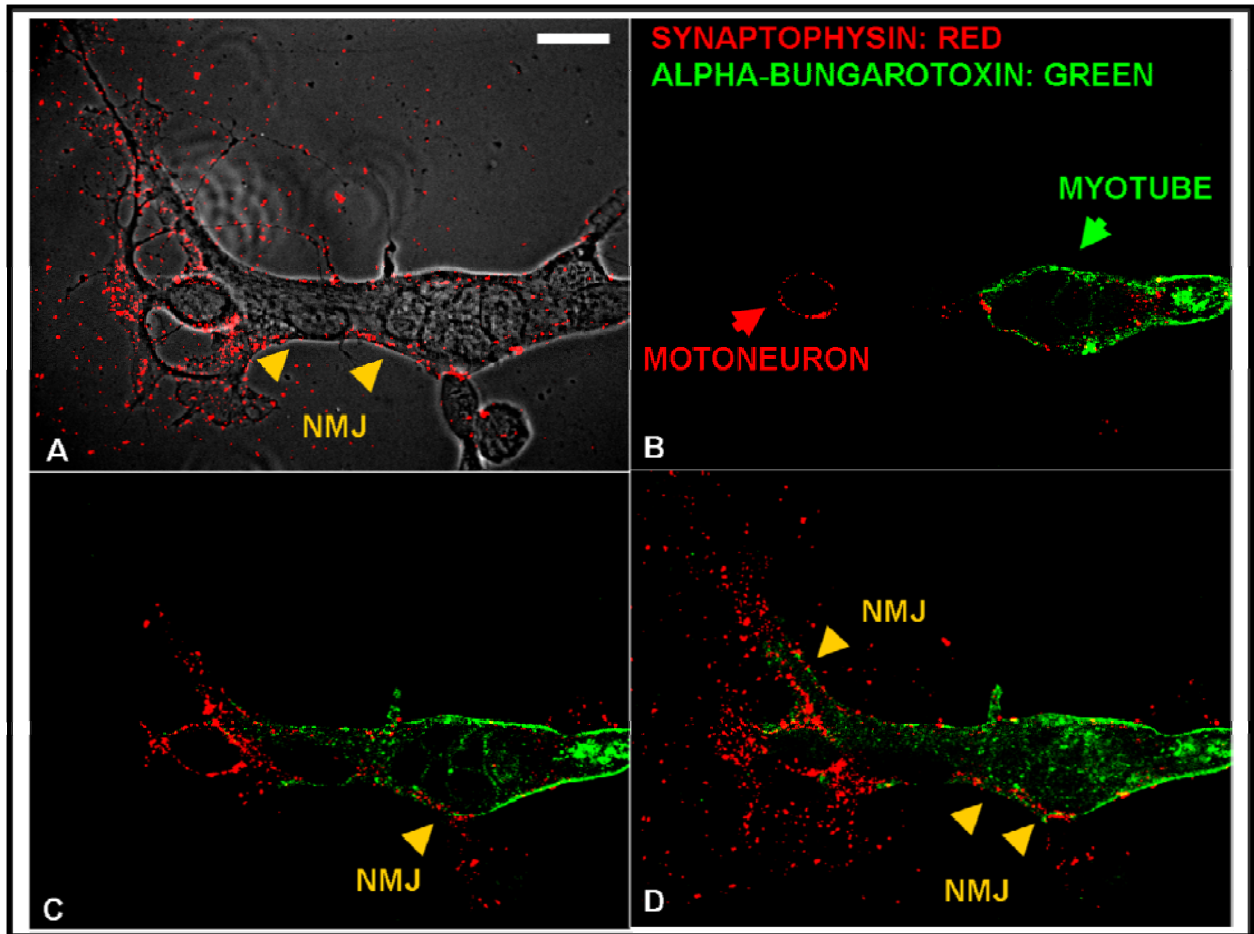


Figure 29. Immunocytochemical evidence for synapse formation at the NMJ. A. Phase picture of a motoneuron in close proximity to a myotube. The motoneuron was stained with synaptophysin (red). B. The motoneuron labeled with synaptophysin (red) and the myotube labeled with alpha-bungarotoxin (green) was observed at a specific plane in the confocal image. C, D. The process of the motoneuron appears to be synapsing on the myotube to form a NMJ in two different planes as seen by the close proximity of the presynaptic marker, synaptophysin (red), and the postsynaptic acetylcholine receptor clustering was indicated by alpha-bungarotoxin labeling (green). Scale bar is 25 μ .

Apart from the neurons and myotubes we observed there were other cells present in the culture which neither manifested myotube nor neuronal morphologies. A large fraction of such cells were mostly individual myocytes which did not fuse to form myotubes. But apart from that we found a small fraction of cells that stained for a glial cell marker (GFAP). We believe that these GFAP positive cells originated from a small fraction of glial cells initially present in the culture and that possibly proliferated in the culture overtime. Two representative pictures of the GFAP positive glial cells are shown in the Figure 30.

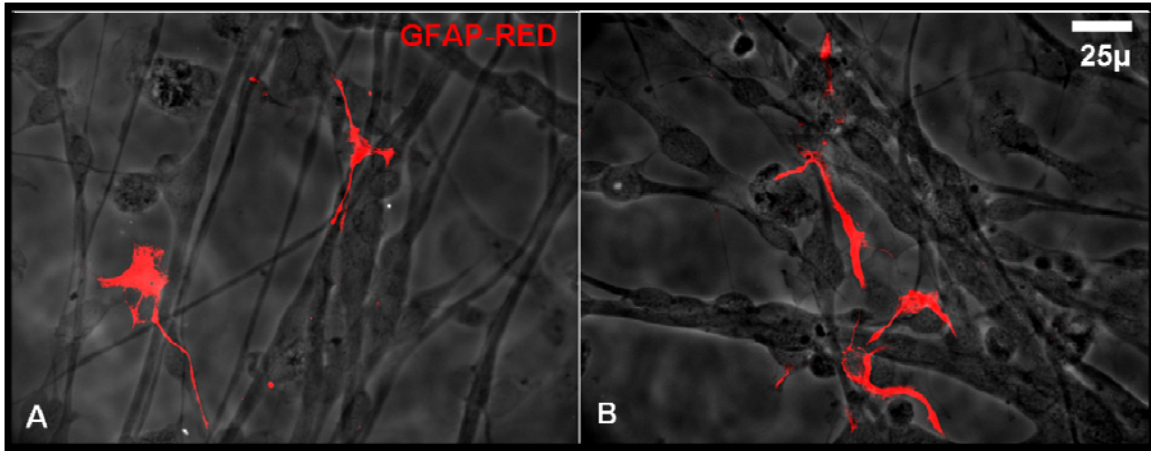


Figure 30. Figure A and B shows the contaminating glial cells (GFAP positive cells are shown in red) present in the co-culture.

We did not find any fibroblast positive cells after staining the culture with mouse anti-human fibroblast monoclonal antibody as seen in Figure 31. In our future studies we will further characterize the role of glial cells in NMJ formation.

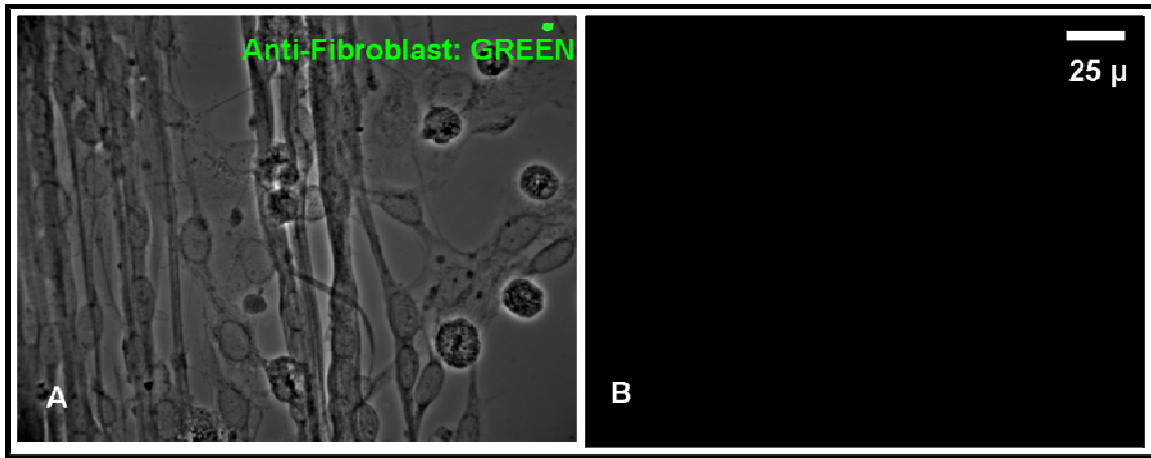
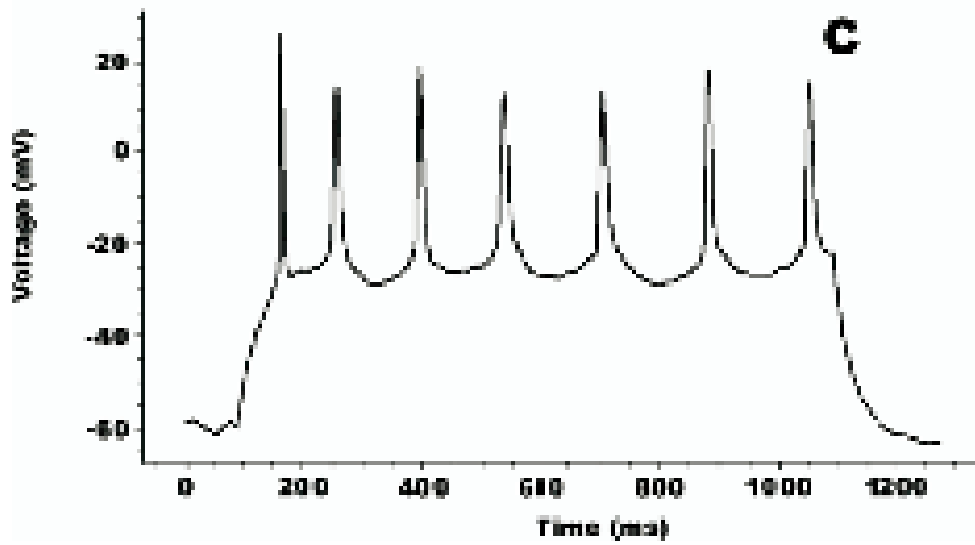
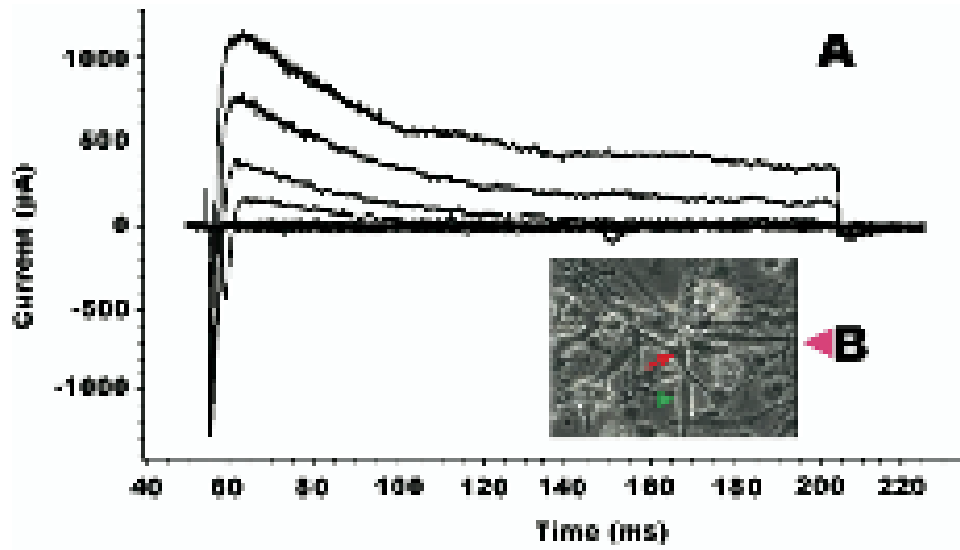


Figure 31. Figure A shows the phase picture of the coculture stained with an anti-fibroblast marker. Figure B indicates the absence of a fibroblast in the coculture after staining with an anti-fibroblast marker (green).

Using patch clamp electrophysiological recordings it was shown that the motoneurons (Figure 32 [A]) as well as the skeletal muscle (Figure 32 [B]) maintained their electrical properties as previously described [11, 12, 27].



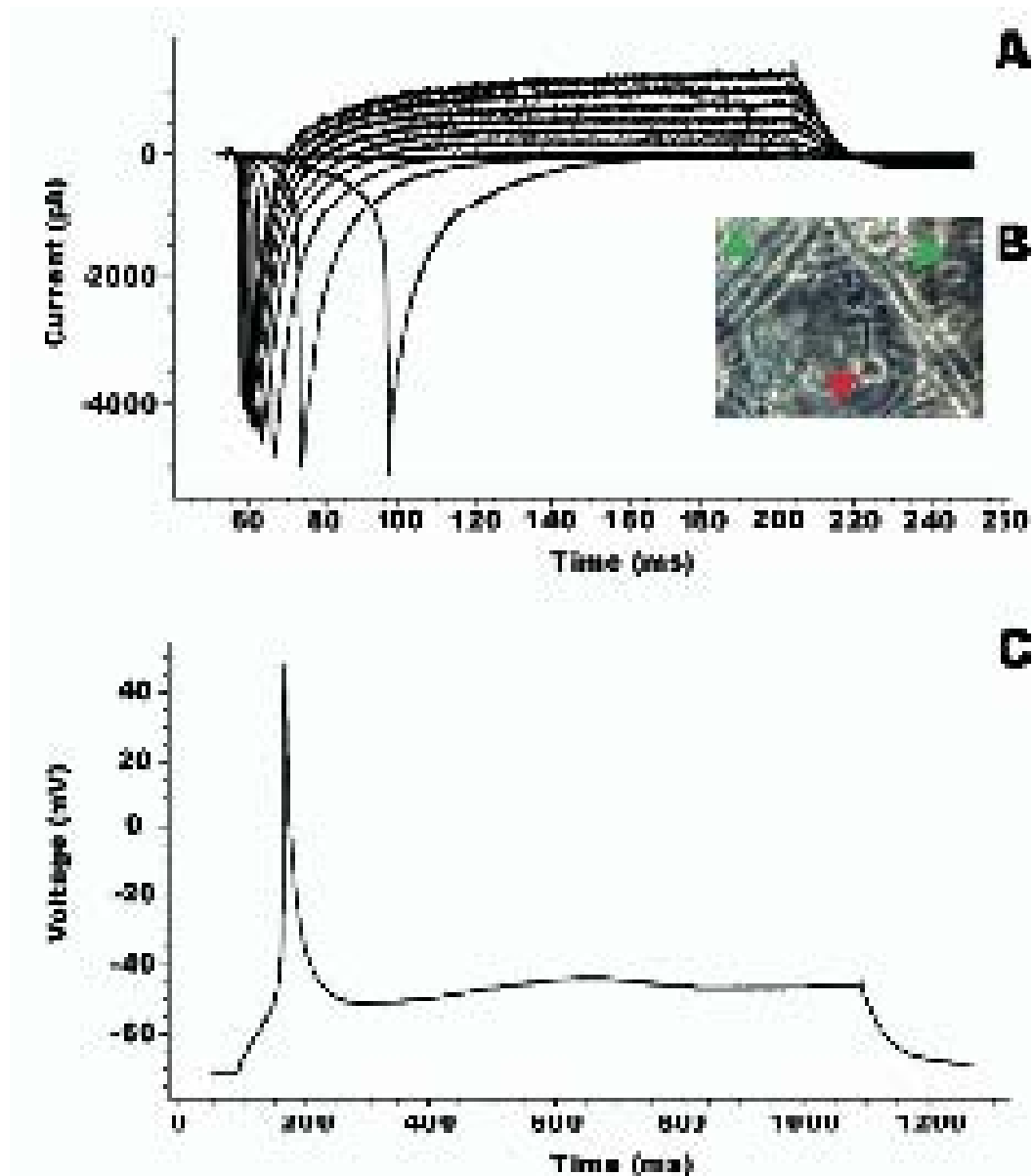


Figure 32.[A]. Electrophysiological properties of a single motoneuron in co-culture. A, voltage clamp recording of the patched motoneuron showing active Na⁺ and K⁺ currents. B, Phase contrast image of patched motoneuron (red arrow) in proximity to a myotube (green arrow). C,

Current clamp recording of the patched motoneuron showing a repetitively firing action potential. These recordings are representative of 95% of the neurons present in the culture.[B]. Electrophysiological properties of a single myotube in co-culture. A. Voltage clamp recording of the patched myotube showing active Na⁺ and K⁺ currents. B, Phase contrast image of patched myotube (green arrow) in proximity to a motoneuron (red arrow). C, Current clamp recording of the patched myotube showing a single action potential. These recordings are representative of 80% of the myotubes present in the culture.

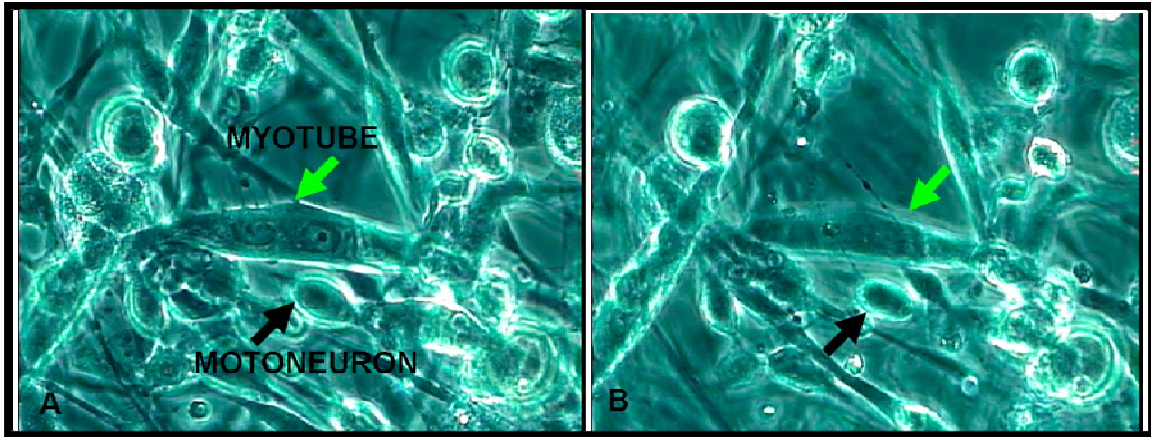


Figure 33. Figure A and B indicate the phase picture of the embryonic motoneuron and myotube pair forming a NMJ. The myotube has been shown to contract rhythmically in Video 2. After the application of the nicotinic cholinergic antagonist d-tubocurarine, the rhythmic contraction of the myotube stopped. Video 2 recorded for 1 minute and 54 seconds. The antagonist d-tubocurarine was applied 45 seconds after the recording started. We observed that the rhythmic contraction of the myotube gradually stopped within the next 10 seconds. Although recordings continued for an additional minute, no further contractions of the myotubes were observed.

Additionally, five representative videographs provide additional evidence for the presence of functional NMJs. All the videos 1, 2, 3, 4 and 5 can be found in the attached disk.

Video 1 shows contracting myotubes surrounding a motoneuron. Coculture of embryonic motoneuron and fetal skeletal muscle at Day 12

Video 2 indicates a myotube contracting rhythmically for the first 45 s. Then d-tubocurarine (also known as curare), an antagonist to the nicotinic acetylcholine receptors present at the NMJ, was added to the recording bath. Soon after the addition of the d-tubocurarine, the rhythmic contraction of the myotube stopped. We recorded for an additional 1 min and 15 s and did not observe any further contractions of the myotube. We concluded that the d-tubocurarine blocked the synaptic transmission from the motoneuron to myotube. 8A and B, indicates the phase pictures of the embryonic motoneuron/myotube pair which formed the NMJ in Video 2 both before and after addition of the d-tubocurarine.

Video 3 was recorded from a co-culture of embryonic motoneuron and fetal skeletal muscle at day 10. Video 3 indicates that the contraction of one myotube in close proximity to a motoneuron was stopped after curare was applied, but the contractions in another myotube distant from the motoneuron continued. In Video 3 d-tubocurarine was applied at 44 s and then the recording continued for another 1 min and 32 s. Video 3 shows that the contraction of one myotube in close

proximity of a motoneuron was stopped after curare was applied, but the contractions in another myotube away from the motoneuron continued due to spontaneous contractions.

Video 4 was recorded from a co-culture of embryonic motoneuron and fetal skeletal muscle at day 10. In Video 4, similar to Video 3, contractions of one myotube stopped after d-tubocurarine application, but the continued contraction of another myotube was noted even after the d-tubocurarine application. The total time for the Video 4 recording was 3 min and 13 s, and d-tubocurarine application occurred after 1 min.

Video 5 was recorded from a co-culture of embryonic motoneuron and fetal skeletal muscle at day 10. Video 5 (control experiment) shows that myotube contractions were not stopped after the application of bath solution without d-tubocurarine. The total time of Video 5 is 5 min and 32 s. Bath solution (without d-tubocurarine) was added at 45 s and the video recording was continued over the next 4 min and 47 s and myotube contractions were observed during the entire time.. This application did not stop the contractions of myotubes.

Videos were recorded from 12 different coverslips which were obtained from two different cell plating experiments. One spot in each coverslip was selected and scanned for myotubes that were contracting in close proximity of a single motoneuron or multiple motoneurons. The recordings were made from the pre-selected spot before and after the application of d-tubocurarine or control

bath solution. Out of the 12 trials, two control experiments were performed where application of the control bath solution without d-tubocurarine was done. The myotube contractions were not stopped after the application of bath solution without d-tubocurarine, as indicated in Video 5. In the 10 remaining trials, d-tubocurarine was applied to the bath solution, and in those experiments distinct effects of d-tubocurarine were observed in three experiments where a contraction of the myotube in close proximity of the motoneuron stopped after d-tubocurarine application. The representative Video 2 shows the distinct effect of d-tubocurarine application on the culture. In the remaining seven trials, it was observed that even after d-tubocurarine application not all the myotubes at the selected spot stopped contracting. Some of the myotubes stopped contracting, whereas others continue to contract. Videos 3 and 4 are representations of the mixed response of the myotubes after d-tubocurarine application. We concluded from these video experiments that the contraction of the myotubes which continued, even after d-tubocurarine application, was due to spontaneous activity, whereas those that stopped contracting after d-tubocurarine application were synapsed to a motoneuron.

Based on the immunocytochemical, electrophysiological and videographed results obtained, this novel culture model provides a controlled, highly reproducible system for the study of neuromuscular junction formation, synaptogenesis and nerve-muscle interactions. Regarding the available literature, this is the first serum-free model system to recreate mammalian NMJs on synthetic substrates in vitro. The advantages of this system are the use of a non-biological

substrate and a defined medium, and it permits controlled system modification, which can further current understanding of the NMJ formation phenomenon. Finally, the ease of DETA substrate patterning will provide a high resolution solution for studying individual neuromuscular junctions, where direct signaling between a neuron and a myotube will be more readily dissected.

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CHAPTER 7: TISSUE ENGINEERING THE NEUROMUSCULAR JUNCTION: A DEFINED MODEL SYSTEM

Introduction

The neuromuscular junction (NMJ), formed between motoneurons and skeletal muscle fibers, is one of the most studied of all synapses. The formation of NMJs *in vivo* is a multi-step process, requiring a highly specialized spatial and temporal interaction of growth factors, hormones and cellular sub-structures, resulting in the formation of a motor endplate. At the motor endplate, a motoneuron terminal (pre-synaptic terminal) interacts with a specialized site on the muscle (post-synaptic terminal) enriched with acetylcholine receptors (AChRs). These specialized sites are called neuromuscular junction (NMJ). In a mammalian vertebrate, whenever an action potential is fired by a motoneuron, it is at these sites that the pre-synaptic vesicles loaded with the neurotransmitter acetylcholine are released in the synaptic cleft. The released acetylcholine molecules diffuse across the synaptic cleft and bind to the post-synaptic terminals in the muscle which are enriched with acetylcholine receptors (AChRs). This leads to depolarization of the muscle cell membrane and eventually to muscle contraction. In this transmission process the electrical impulses (action potentials) generated by the motoneuron are converted to chemical signals. Next, the chemical signal is converted into a mechanical signal in the form of muscle contraction. Therefore, not only do NMJs represent an important system for studying synapse

formation and maturation, but also for studying how cells interconvert messages between electrical, chemical and mechanical modalities[1-4].

In vitro culture models are powerful cell biology tools for studying the role of different growth factors, hormones and cellular structures relevant to NMJ formation. Furthermore, a system utilizing defined media and growth substrates makes it easier to understand the effects of manipulating specific factors within the system. However, very little research, had been undertaken in developing cell biological techniques for making mammalian NMJs in the presence of such a system. This lack of information in cell biological technique is a stumbling block in successfully tissue engineering the NMJ[1, 2]. The successful creation of a tissue engineered NMJ construct, requires the development a defined medium and well characterized synthetic biomaterial promoting NMJ formation. Recently, we attempted to create a tissue engineered construct of NMJ on a biocompatible silane substrate (DETA) using a defined medium formulation. Though we were successful in tissue engineering NMJs, the model represented a proof of concept design[5]. Consequently, areas of design improvement were identified and became the focus of further research.

This manuscript details the following design improvements to the current model: 1) increasing the longevity of the nerve-muscle co-culture forming NMJs, 2) developing mature phenotypes of muscle forming NMJs, 3) increasing the number of NMJs in culture. Using an additional group of

growth factors known to be critical for neuromuscular junction formation and maturation, we have developed a more robust, long-term model for co-culturing embryonic rat motoneurons and fetal rat skeletal muscle. The cultures have been characterized morphologically and immunocytochemically. Furthermore, the formation of neuromuscular junctions has been demonstrated using extensive immunocytochemistry. This model will find extensive applications in basic research in NMJ, tissue engineering NMJ, bio-hybrid device development for limb prosthesis and in regenerative medicine.

Methods and Materials

Surface modification

Glass coverslips (Thomas Scientific 6661F52, 22×22 mm No. 1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 min at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) film was formed by the reaction of the cleaned surface with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by XPS (Kratos Axis 165) by monitoring the N 1s peak[5-13].

Rat embryonic motoneuron isolation

Rat spinal motoneurons were purified from ventral cords of embryonic day 14 (E14) embryos. Briefly, rats were anaesthetized and killed by inhalation of an excess of CO₂. This procedure was in agreement with the Animal Research Council of University of Central Florida, which adheres to IACUC policies. Ventral spinal cells from the embryo were collected in cold Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27. The cells were dissociated with 0.05% trypsin-EDTA (Invitrogen) treatment for 15 minutes. The dissociated cells were layered over a 4 ml step gradient (Optiprep diluted 0.505: 0.495 (v/v) with Hibernate E/ GlutaMAX™ / antibiotic antimycotic/ B27 and

then made to 15%, 20%, 25% and 35% (v/v) in Hibernate E/ GlutaMAX™/ antibiotic-antimycotic/ B27) followed by centrifugation for 15 min, using 250 g at 4°C. This is modified from the previously described protocols due to non-availability of metrizamide. After centrifugation, four bands of cells were obtained. The motoneurons constituted the uppermost band with large somas. These cells present in the uppermost band were collected in fresh Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27 and centrifuged for 5 minutes at 200 g and at a set temperature of 4°C. The pellet formed contains the motoneuron, and these are further used for culture[10]. Purified motoneurons were plated on top of muscle cells at a density of 100 cells/mm². Motoneuron plating was done 30 minutes after plating the muscle cells.

Muscle cell isolation and culture

The skeletal muscle was dissected from the thighs of the rat fetus (E17) hind limbs. The tissue was collected in a sterile 15 ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 2 ml of 0.05% of trypsin-EDTA solution for 60 min in a 37 °C water bath (100 rpm). After 60 min the trypsin solution was removed and 4 ml of Hib. E +10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated and the supernatant transferred to a 15 ml centrifuge tube. The same process was repeated two times by adding 4 ml of Hib. E +10% FBS each time. The 12 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in Hib. E medium) cushion and

centrifuged at 300g for 10 min at 4 °C. Finally, the cell pellet was washed three times with Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27 and re-suspended in 10 ml of Hibernate E/ GlutaMAX™ / antibiotic-antimycotic/ B27 and plated on 100 mm uncoated dishes for 30 min. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and plated at a density of 700–1000 cells/mm² on DETA coated coverslips. After 30 minutes motoneurons were plated on top of the muscle cells. The cells attached to the substrate in 1 h. After 1 h, serum free medium was added to the culture wells containing the coverslips. The cultures were maintained at 37°C in a 5% CO₂ incubator (relative humidity 85%)[9, 13].

Serum-free medium composition and medium change technique

Serum-free medium composition was described in table 8 and table 9. Medium change technique was described in figure 34. The culture model was optimized after more than fifty trials. The current data was generated from 25 trials (n=25).

Table 8: Composition of medium 1.

S. No	Component	Amount	Catalogue #	Source	References
1.	Neurobasal A	500 ml	10888	Gibco/ Invitrogen	[14]
2.	Antibiotic-Antimycotic	5 ml	15240-062	Gibco/ Invitrogen	
3.	Glutamax	5 ml	35050-061	Gibco/ Invitrogen	
4.	B27 Supplement	10 ml	17504-044	Gibco/ Invitrogen	[11, 14]
5.	G5 Supplement 100X	5 ml	17503-012	Gibco/ Invitrogen	[15-24]
6.	VEGF ₁₆₅ r Human	10 µg	P2654	Gibco/ Invitrogen	[25-28]
7.	Acidic FGF	12.5 µg	13241-013	Gibco/ Invitrogen	[15, 22, 24, 29-34]
8.	Heparin Sulphate	50 µg	D9809	Sigma	[15, 22, 24, 29-34]
9.	LIF	10 µg	L5158	Sigma	[35-43]
10.	Vitronectin (Rat Plasma)	50 µg	V0132	Sigma	[44, 45]
11.	CNTF	20 µg	CRC 401B	Cell Sciences	[46-50]
12.	NT 3	10 µg	CRN 500B	Cell Sciences	[51]
13.	NT 4	10 µg	CRN 501B	Cell Sciences	[52, 53]
14.	GDNF	10 µg	CRG 400B	Cell Sciences	[54-58]
15.	BDNF	10 µg	CRB 600B	Cell Sciences	[53, 59, 60]
16.	CT-1	10 µg	CRC 700B	Cell Sciences	[61-69]

Table 9: Composition of medium 2

S. No	Component(s)	Amount	Catalogue	Source	References
1.	Neurobasal A	500 ml	10888	Invitrogen/Gibco	[14]
2.	Glutamax	5 ml	35050-061	Invitrogen/Gibco	
3.	Antibiotic-antimycotic	5 ml	15240-062	Invitrogen/Gibco	
4.	B27 supplement	10 ml	17504-044	Invitrogen/Gibco	[11, 14]
5.	Cholesterol (250X)	5ml	12531	Invitrogen/Gibco	[70]
6.	TNF-alpha, human	10 µg	T6674	Sigma-Aldrich	[71-73]
7.	PDGF BB	50 µg	P4056	Sigma-Aldrich	[35, 74-77]
8.	Vasoactive intestinal peptide (VIP)	250 µg	V6130	Sigma-Aldrich	[78]
9.	Insulin-like growth factor 1	25 µg	I2656	Sigma-Aldrich	[41, 42, 72]
10.	NAP	1mg	61170	AnaSpec, Inc.	[79, 80]
11.	Recombinant Apolipoprotein E2	50 µg	P2002	Panvera, Madison, WI	[81]
12.	Laminin, mouse purified	2 mg	08-125	Millipore	[82-88]
13.	Beta amyloid (1-40)	1mg	AG966	Millipore	[89-91]
14.	Human Tenascin-C protein	100 µg	CC065	Millipore	[92]
15.	rr- Sonic hedgehog, Shh N-terminal	50 µg	1314-SH	R&D Systems	[93-104]
16.	Rr (Agrin C terminal)	50 µg	550-AG-100	R&D Systems	[105, 106]

Figure 34. The modified protocol for co-culturing motoneuron and skeletal muscle.

Immunocytochemistry

Neonatal myosin heavy chain (Neonatal MHC)

Coverslips were rinsed with PBS, fixed in 20 °C methanol for 5–7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution), and blocked for 30 min in a permeabilization solution + 10% goat serum (blocking solution). Cells were incubated overnight with primary antibody against neonatal MHC (N3.36, IgG, Developmental Studies Hybridoma Bank) diluted (1:5) in the blocking solution. Cells were washed with PBS and incubated with the secondary antibody (diluted in PBS) for 2 h. The secondary antibody solution was removed and the cells were rinsed using PBS. The coverslips were dried and mounted on glass slides using VectaShield+DAPI mounting medium (Vector Laboratories H-1200) and viewed on a confocal microscope (UltraVIEW™ LCI, PerkinElmer)[5, 107, 108]. Immunocytochemistry results were verified in three different experiments.

Double staining with Neurofilament 150 and Neonatal myosin heavy chain

Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4°C with rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:2000) and neonatal MHC (N3.36, IgG, Developmental Studies Hybridoma Bank diluted 1:5). After overnight incubation, the coverslips were rinsed three times with PBS and then incubated with the appropriate secondary antibodies for 2h. After rinsing three times in PBS, the coverslips were mounted with Vectashield+DAPI mounting medium onto glass

slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative[5]. Immunocytochemistry results were verified in three different experiments.

AChR labeling, AChR + Synaptophysin co-labeling and observation in co-cultures

AChRs were labeled as described previously by incubating cultures with 5×10^{-8} M of α -bungarotoxin, Alexa Fluor® 488 conjugate (Molecular Probes, B-13422) for 1.5 hr at 37°C before observation. Labeled cultures were fixed with glacial acetic acid and ethanol, washed with PBS, dried, mounted and examined by confocal microscopy. The coverslips which were used for double staining with AChR + Synaptophysin for locating the NMJ were processed further. After 1.5 hr of α -bungarotoxin labeling of AChR receptors, the coverslips were fixed, blocked, permeabilized and incubated overnight with synaptophysin antibody (MAB368, diluted 1:1000; Millipore/Chemicon), the pre-synaptic marker present on the motoneurons. Synaptophysin is a major synaptic vesicle protein, p38, carrying the neurotransmitters[5, 109].

Results and Discussion

DETA Surface Modification

DETA modified surface supported long-term survival of the co-culture. Since the silane forms a uniform monolayer hence the cells distribute evenly upon plating. The formation of monolayer was verified using x-ray photoelectron spectroscopy (XPS) and contact angle measurement techniques. The results were similar as reported previously [5, 7-13]. The use of synthetic substrate offers a scope to dissect the role of different extracellular matrix proteins involved in NMJ formation. We are attempting to pattern the substrate in different geometries in order to orient the myotubes and the motoneurons in specific directions. This patterning will assist in studying NMJs in a much more controlled manner and will make it easy to study the electrical activities in a local environment.

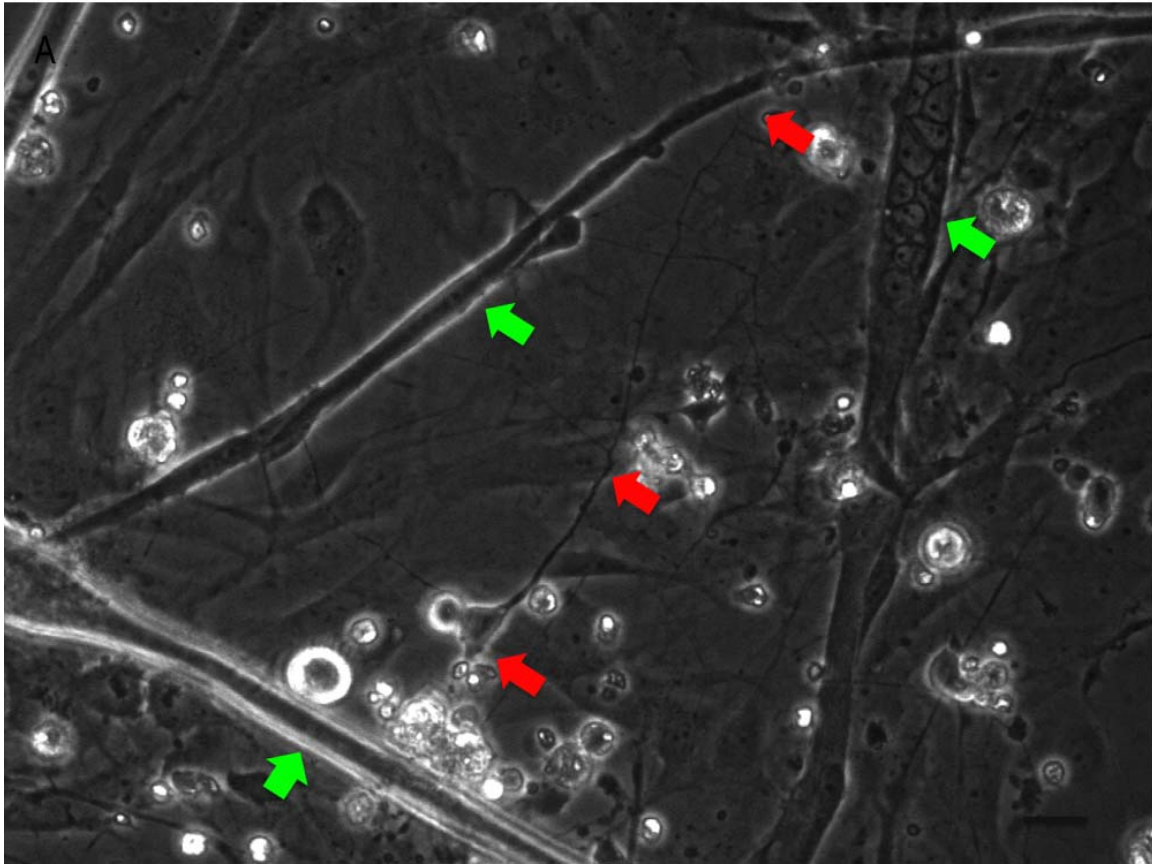
Temporal growth factor application

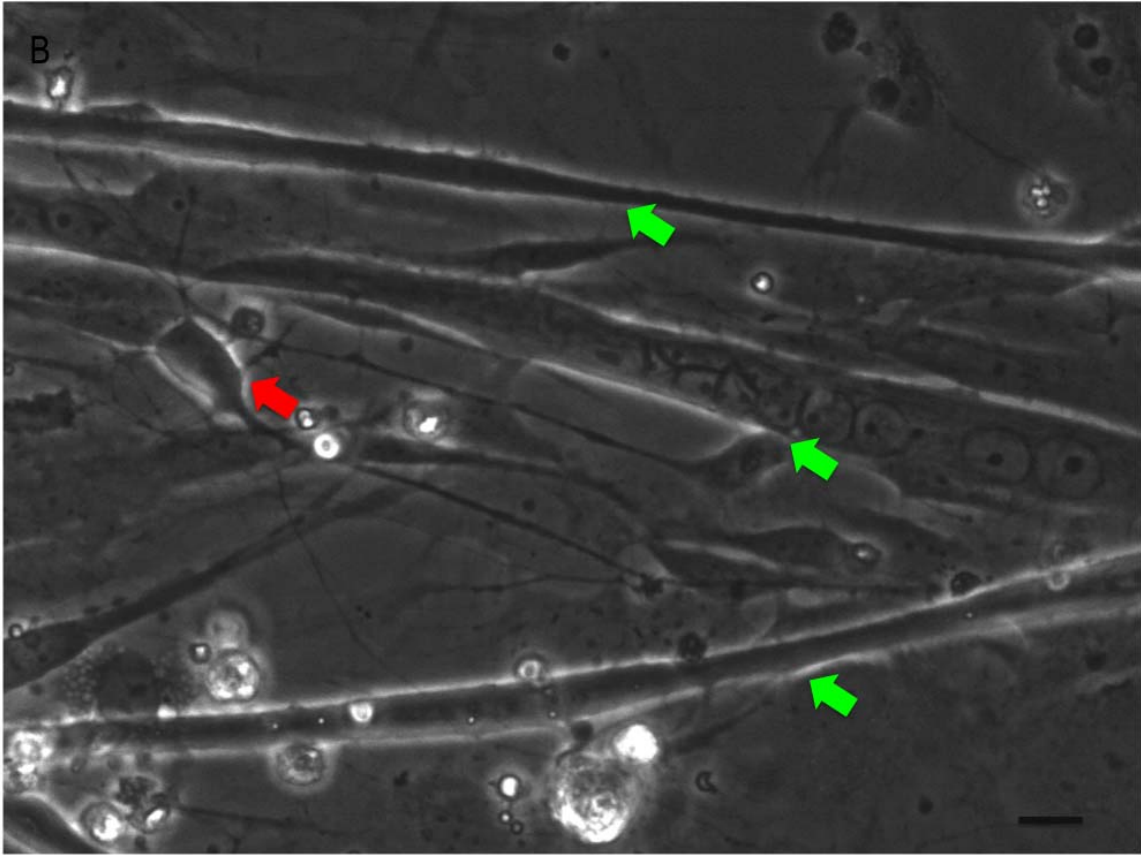
The formation of the maximal number of neuromuscular junctions was observed using the temporal growth factor application technique described in Figure 34. Basically, upon plating of motoneurons and skeletal muscle, the cells were given fed with a medium containing factors to promote both growth and survival as well as to enhance NMJ formation (Table 8 and Table 9). After 3 days in culture, the whole medium was removed and was switched to a minimal formulation, NB4Activ, which facilitated long-term survival and further development of NMJs (Figure 34). This media (Table 8 and Table 9) were formulated empirically. We had provided the references from the

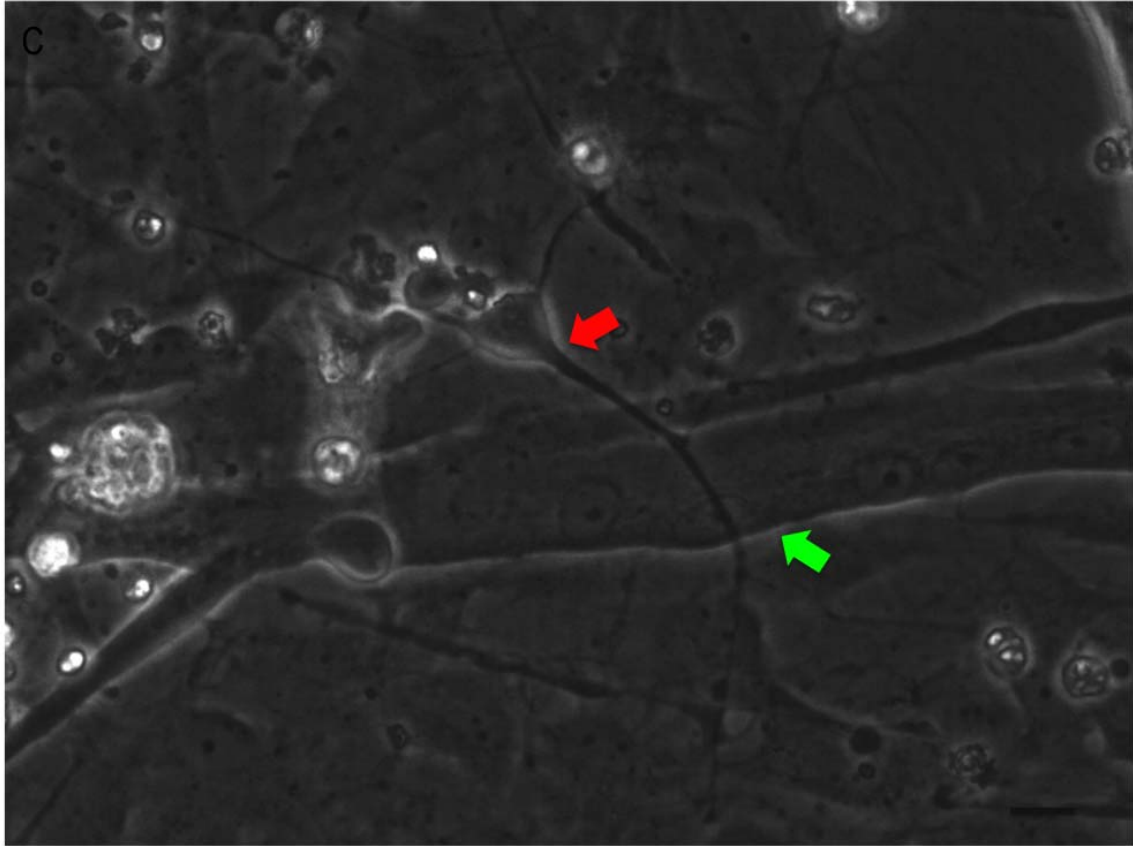
available literature which indicated that these different factors are involved in motoneuron, muscle survival as well as in NMJ formation.

Morphology of the cultures showing interaction of motoneurons and skeletal muscle myotubes

Using phase contrast microscopy, we were able to visualize motoneuron axons appearing to interact with skeletal muscle myotubes between day 12-15 (Figure 35 A-D). Some of the axonal processes also appear to branch and terminate on the myotubes.







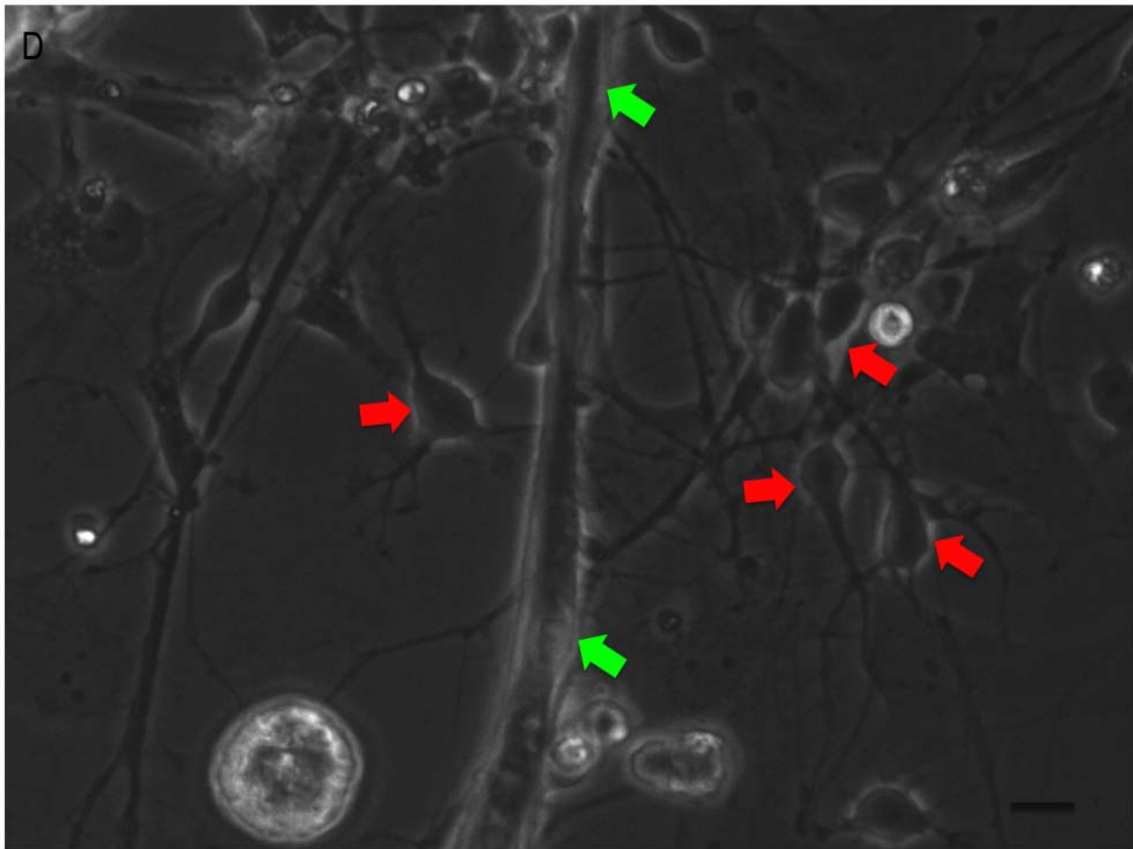
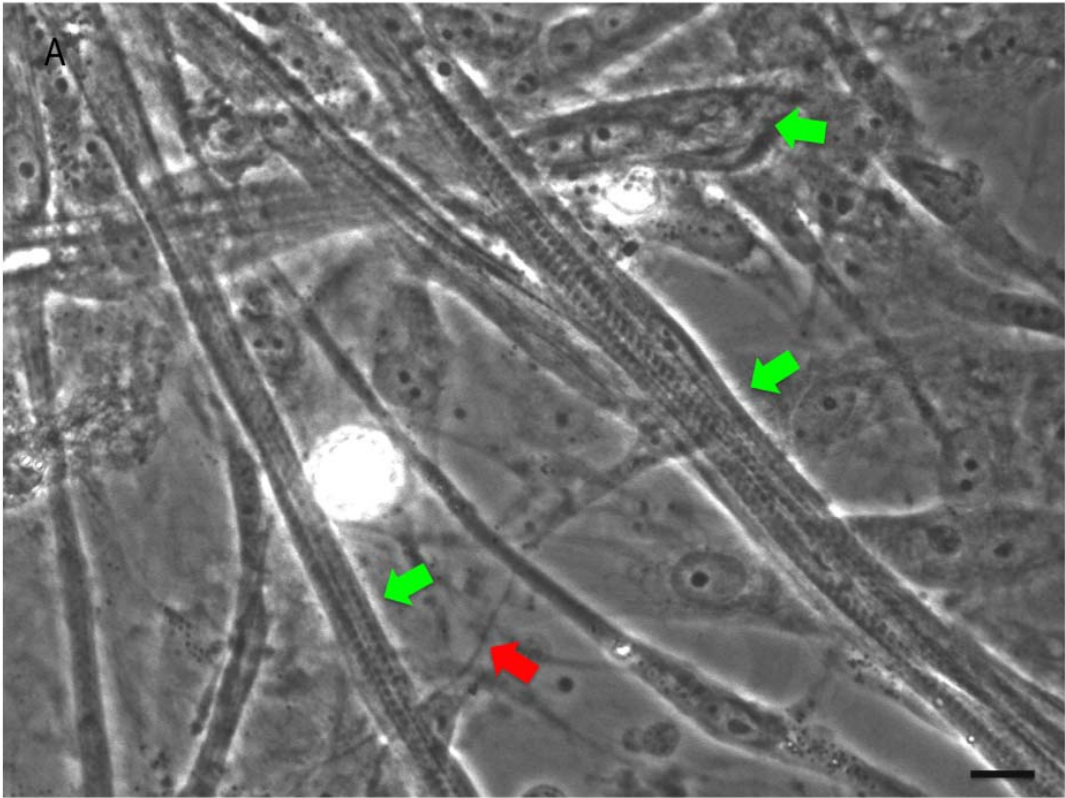
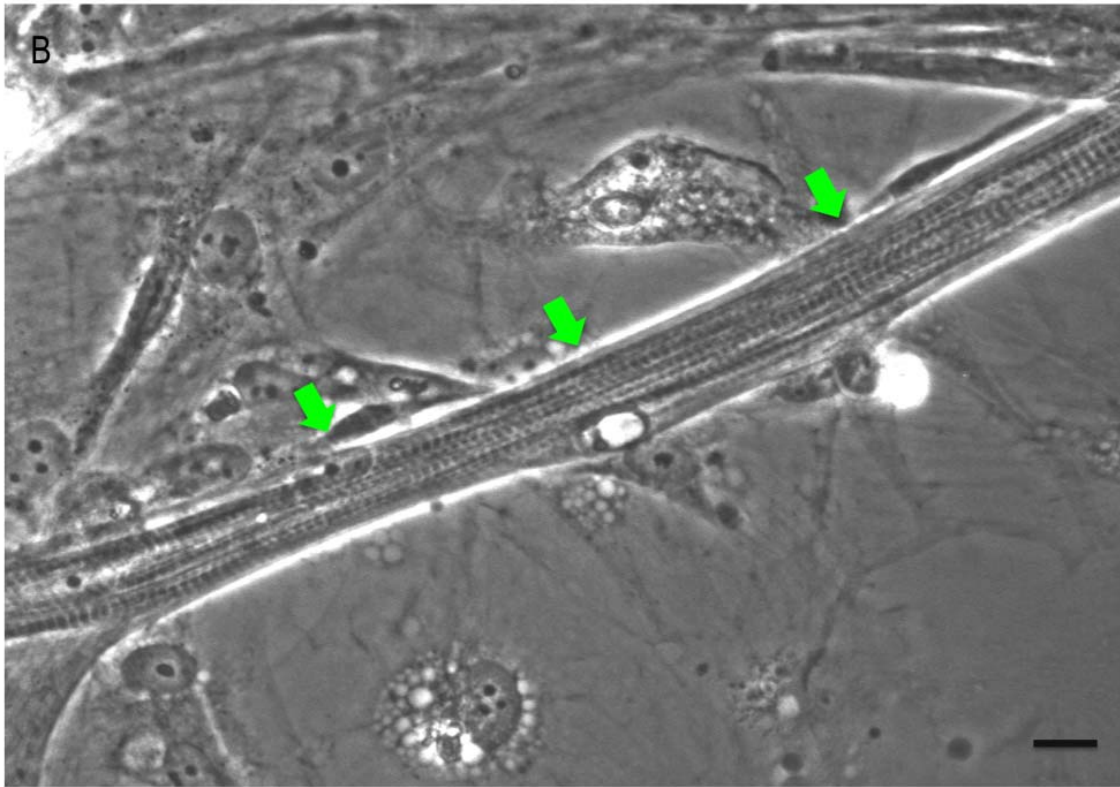
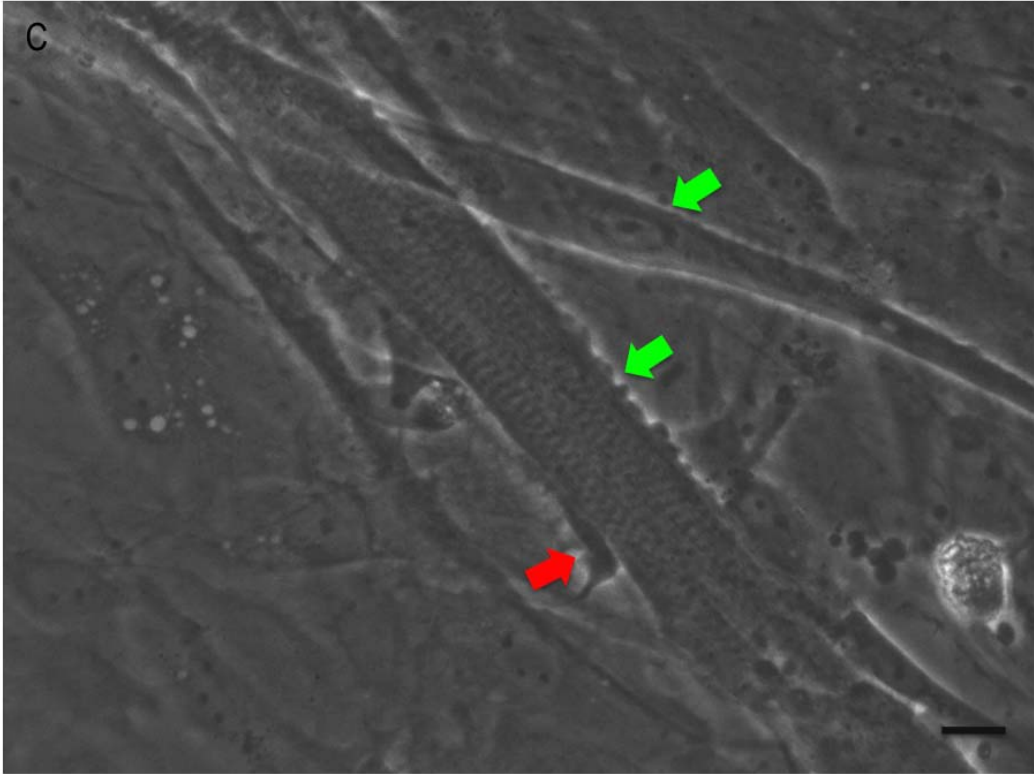


Figure 35. A-D: Phase contrast pictures of the motoneurons and skeletal muscle in co-culture between day 12-15. The distinct morphology of motoneuron and the processes were indicated by red arrows. The myotubes were shown by green arrows. The scale bar is 25 μ .

Furthermore, many of the myotubes exhibited the characteristic striation pattern observed after sarcomere formation when the fibers reached approximately 25-30 days in culture (Figure 36 A-D).







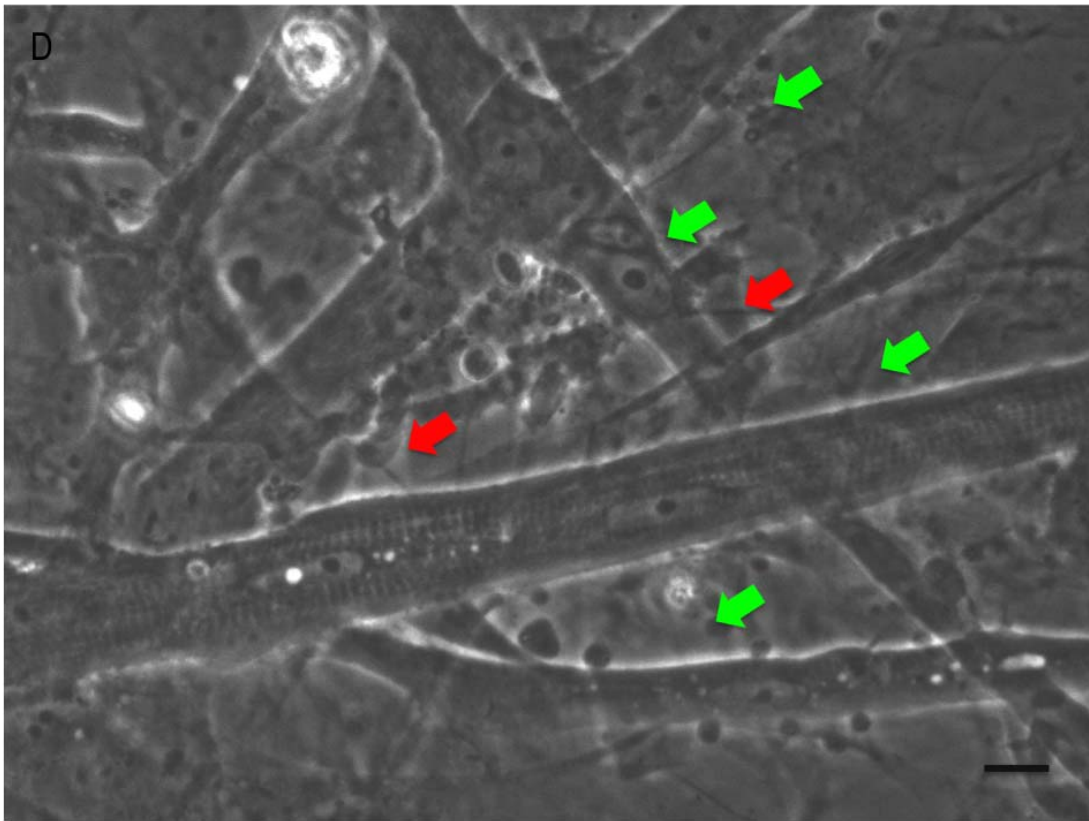
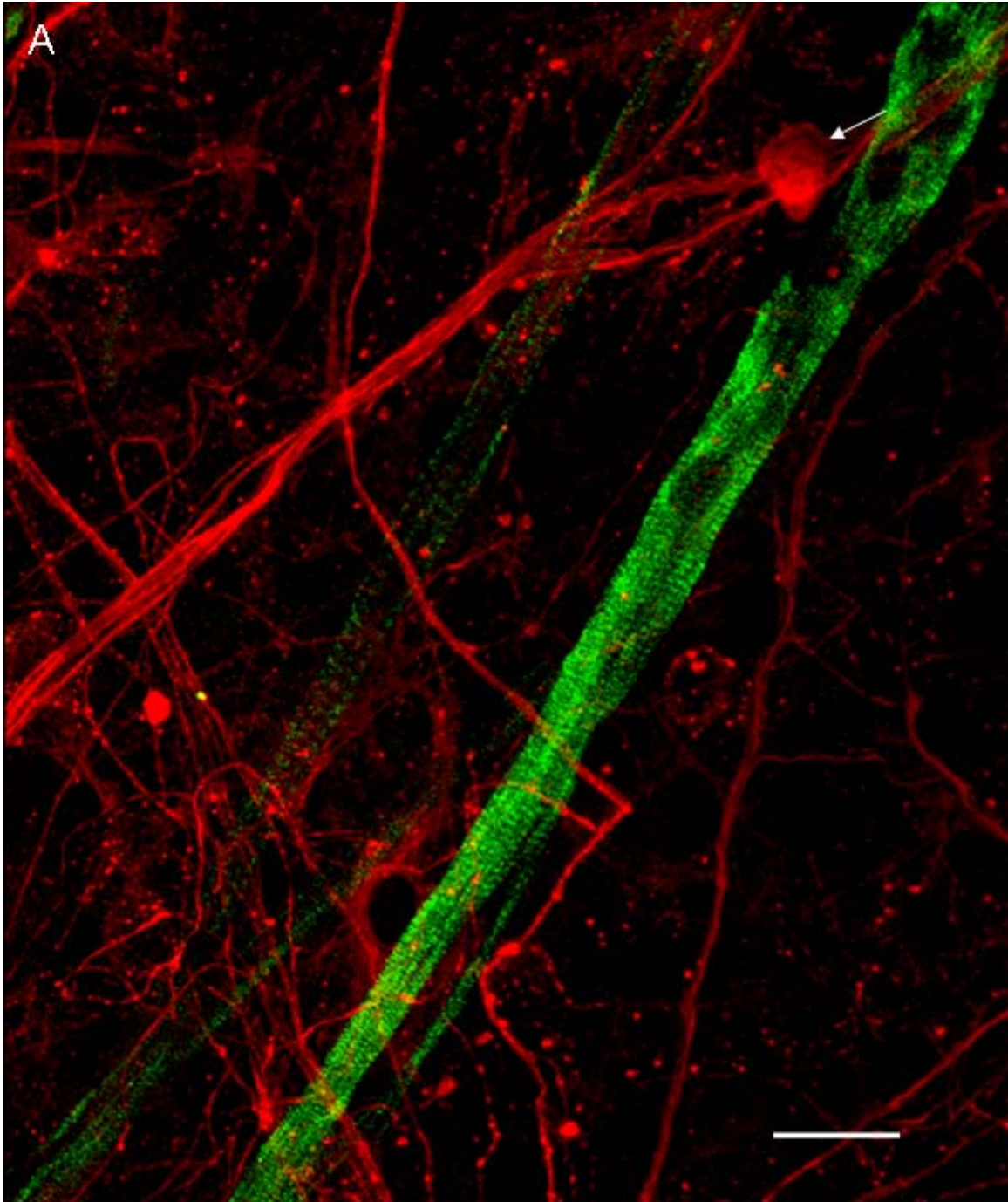


Figure 36. Phase contrast pictures of the co-cultures between day 25-30. A,B: The myotubes showing characteristic striations. C,D: Panels showing myotubes with striations and myotubes without striations. The red arrows indicate the neuron cell body and the processes. The green arrow indicated the myotubes. The scale bar for A, B is 40 μ . The scale bar for C, D is 25 μ .

Immunocytochemical characterization of motoneuron and skeletal muscle in culture

Next, we wanted to evaluate the characteristic protein expression pattern of the motoneurons and myotubes in co-culture. We used immunocytochemistry to visualize neurofilament protein expression in the motoneurons and neonatal myosin heavy chain (MHC) for myotube visualization (Figure 37 A-B). The motoneuron processes were seen all over the myotubes. The characteristic striations of the myotubes were more visible after staining with neonatal myosin heavy chain antibody.



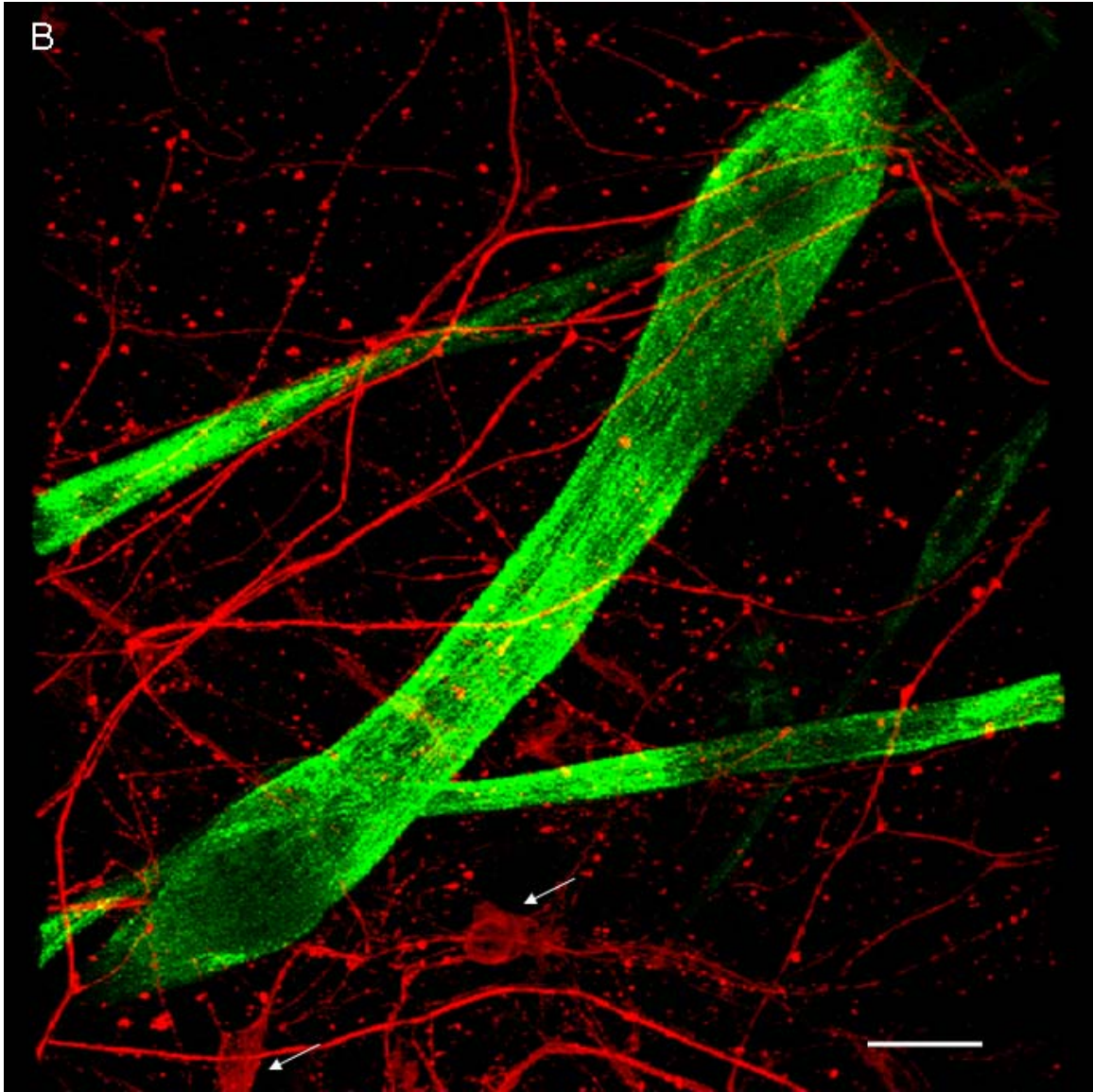
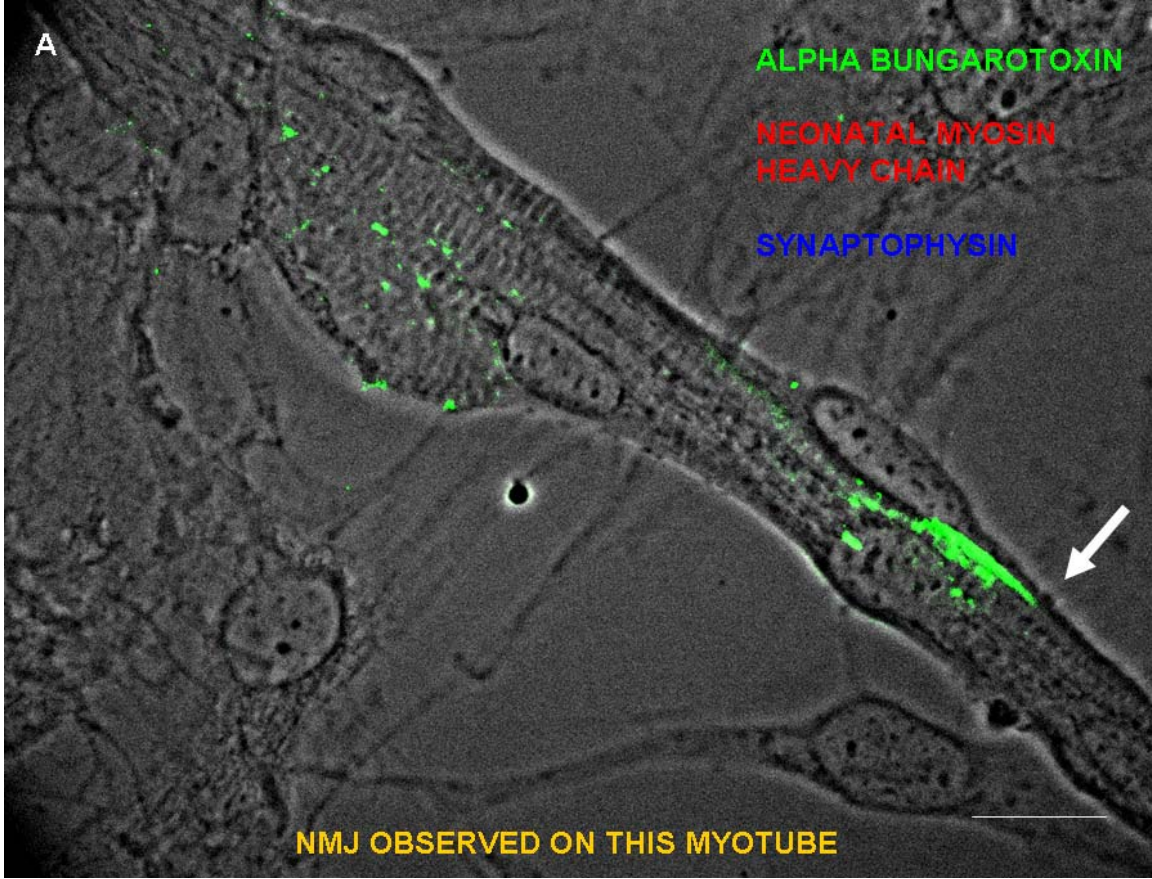
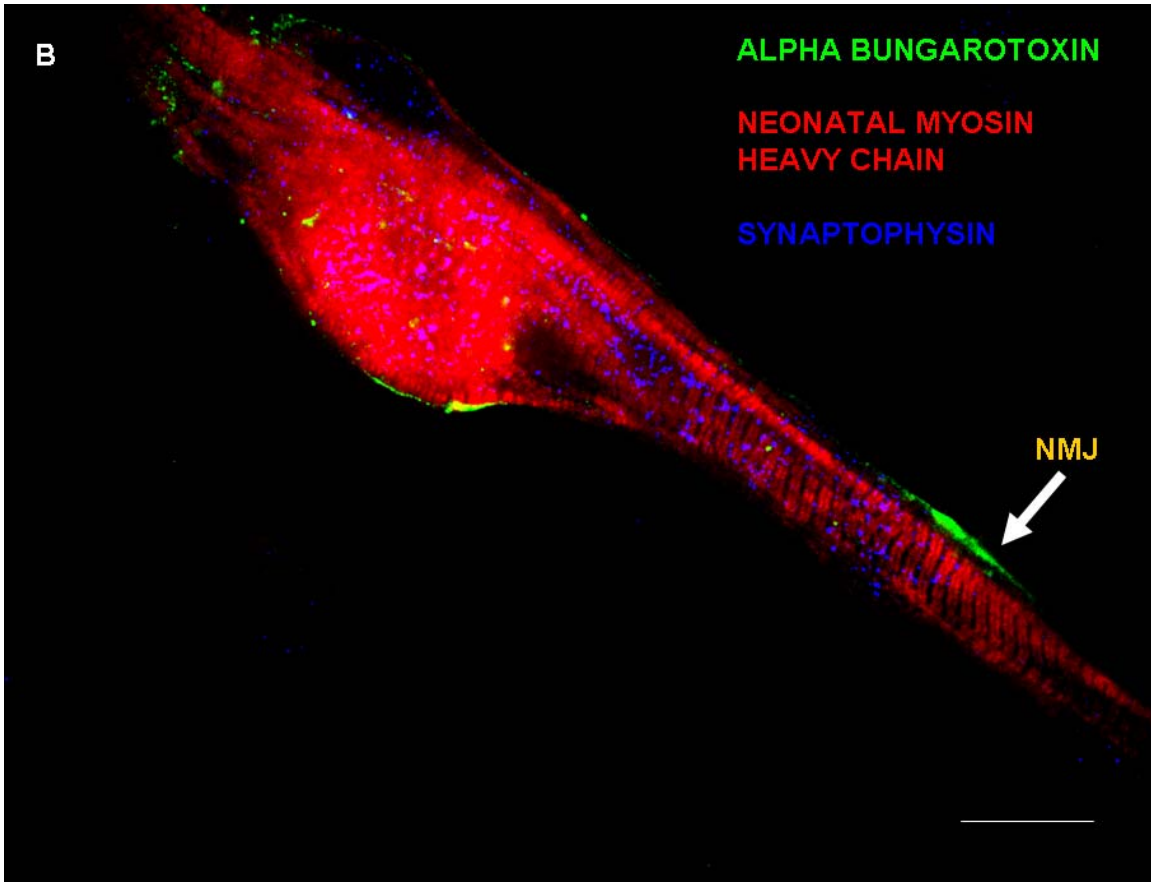


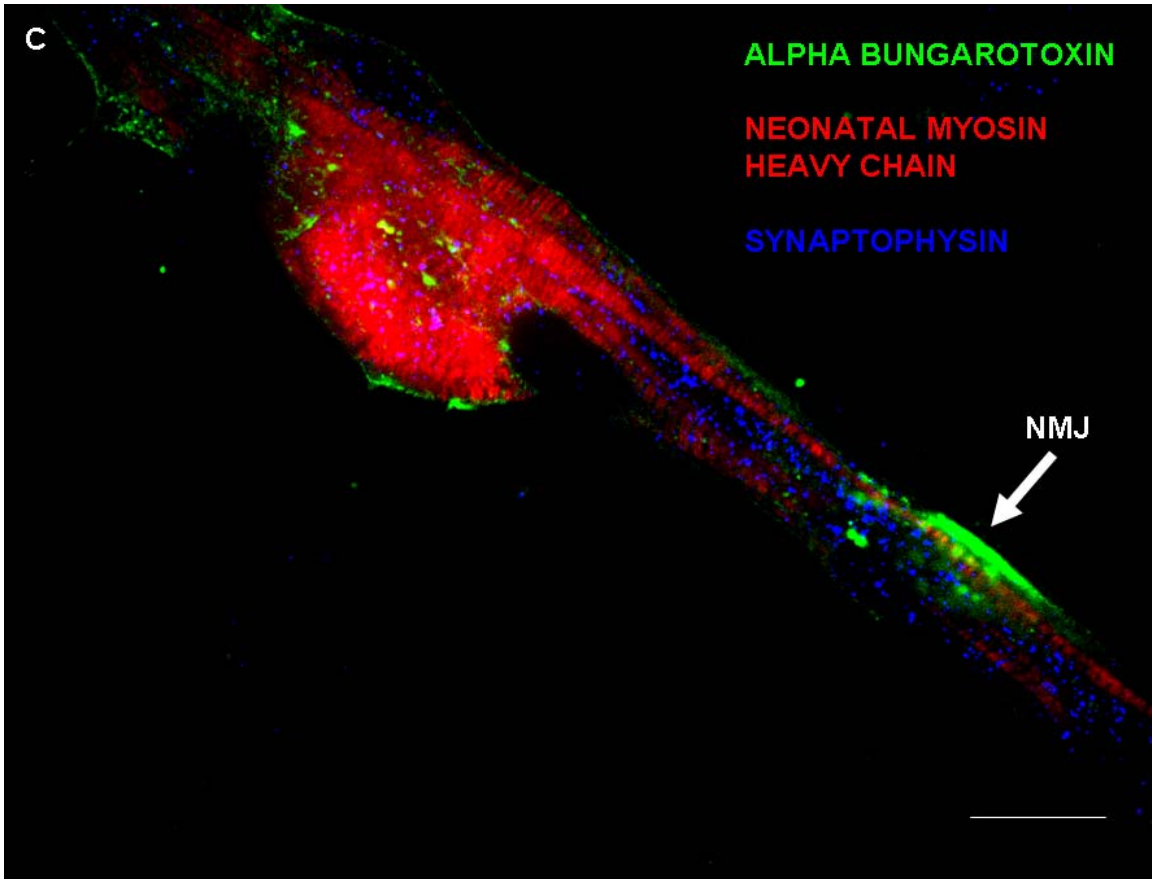
Figure 37. A,B: Cocultures at day 25 were stained with NF-150 and N3.36. NF-150 (red) stained the large motoneurons and their processes. The motoneurons were shown by white arrows. The myotubes (green) stained for N3.36. The neuronal processes were seen all over the myotubes. The characteristic striations of the myotubes were observed in the pictures. The scale bar was 50 μ .

Neuromuscular junction formation

In order to determine neuromuscular junction formation using this new medium formulation, we analyzed the clustering of AChRs using alpha bungarotoxin and their colocalization with synaptophysin vesicles. The colocalization of these two synaptic structures indicates the proximity of pre-synaptic and post-synaptic structures and is a good indication of NMJ formation. Using this technique, we were able to identify the colocalization of synaptophysin vesicles with AChR clusters (Figure 38 A-D).







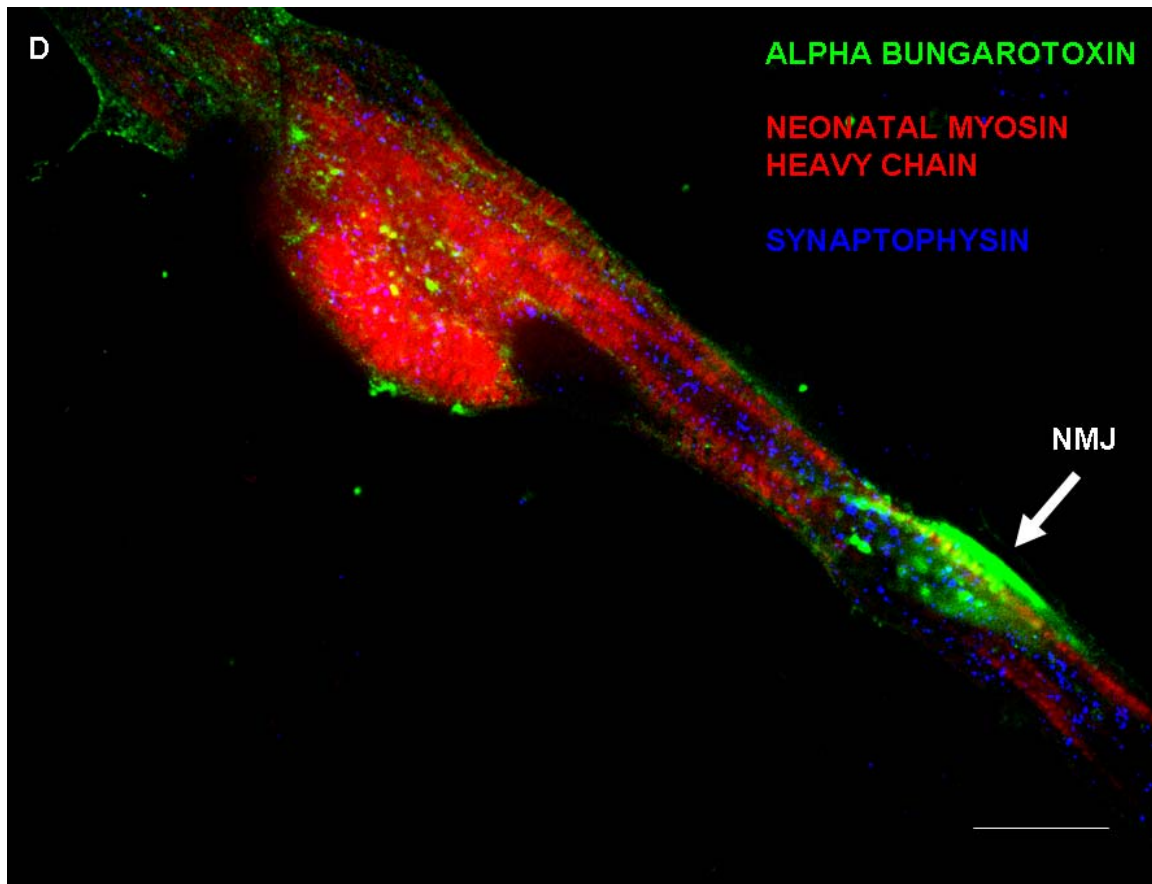
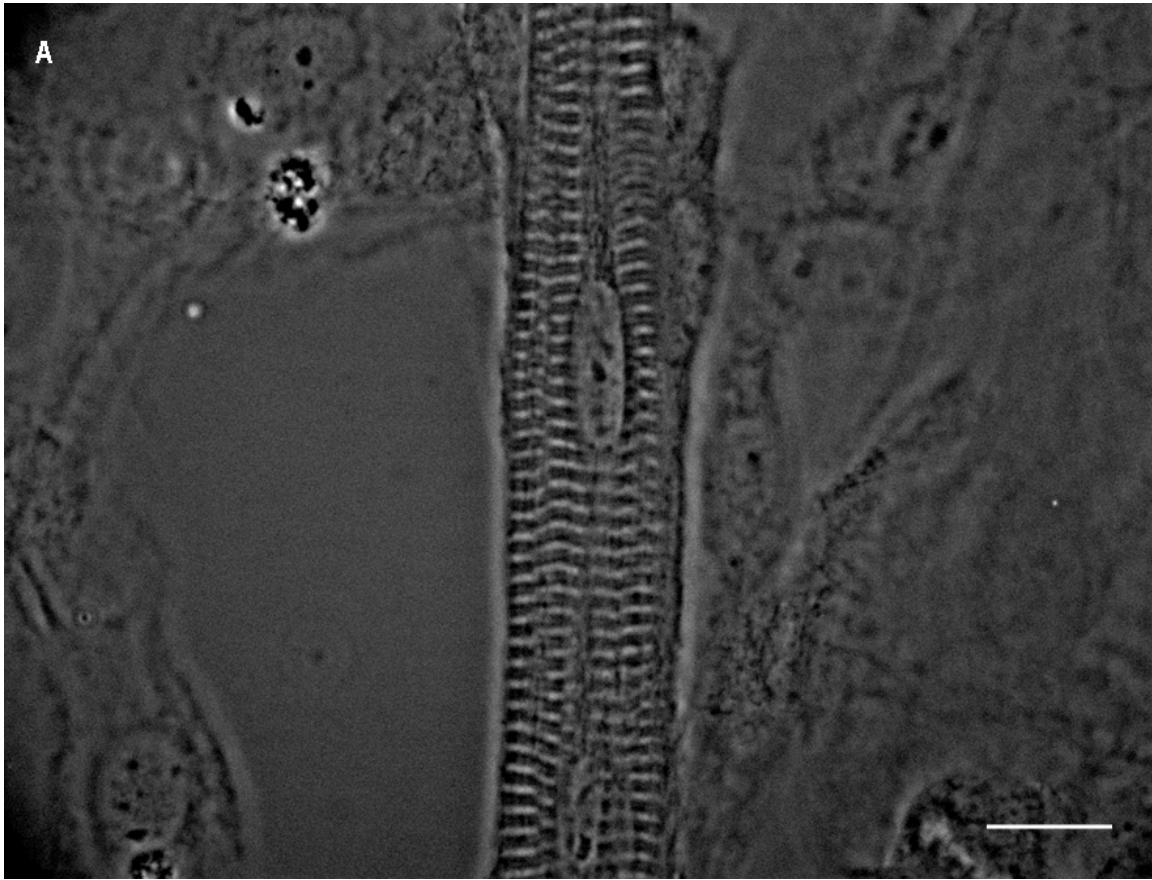


Figure 38.A-D: Neuromuscular Junction (NMJ) formation between day 30-40. The embryonic motoneuron-skeletal muscle coculture were stained with neonatal myosin heavy chain (N3.36) antibody (Red), alpha-bungarotoxin (post-synaptic marker labeled with green) and synaptophysin (pre-synaptic marker labeled with blue). Colocalization of pre-synaptic and post-synaptic markers indicate the synapse formation at NMJ. A. Phase picture of the myotube showing the alpha-bungarotoxin staining in green. B. Triple stain, showing the close proximity of alpha-bungarotoxin (green) and synaptophysin (blue) indicating synapse formation at a specific plane and the striations of the muscle were seen in red (NMHC). C-D. Observing the NMJ at two different planes using the confocal microscopy. A much more dense clustering of synaptophysin and alpha-bungarotoxin was observed at these planes.

We also identified axon + myotube interactions that did not result in the colocalization of pre-synaptic and post-synaptic structures (Figure 39 A-B). The observation of the negative result defines the difference between colocalization and non-colocalization emphasizes the positive observed in this system.



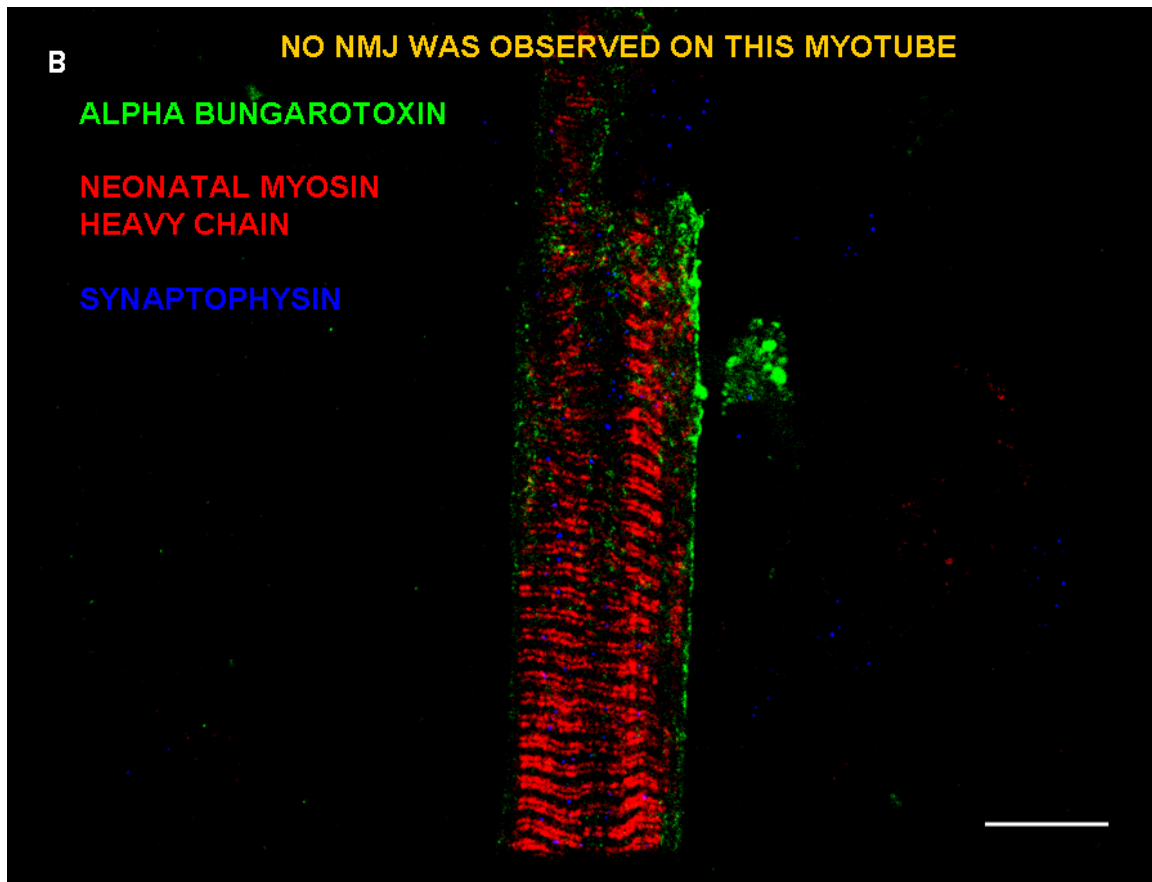
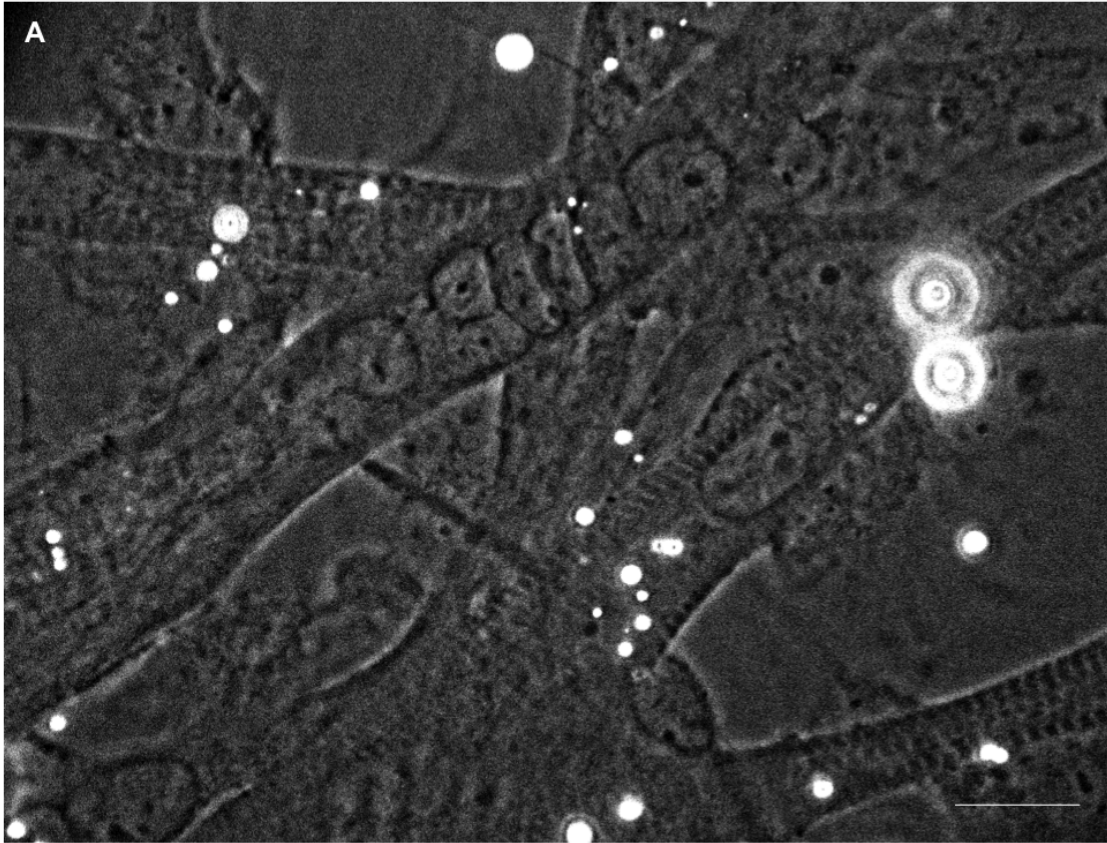
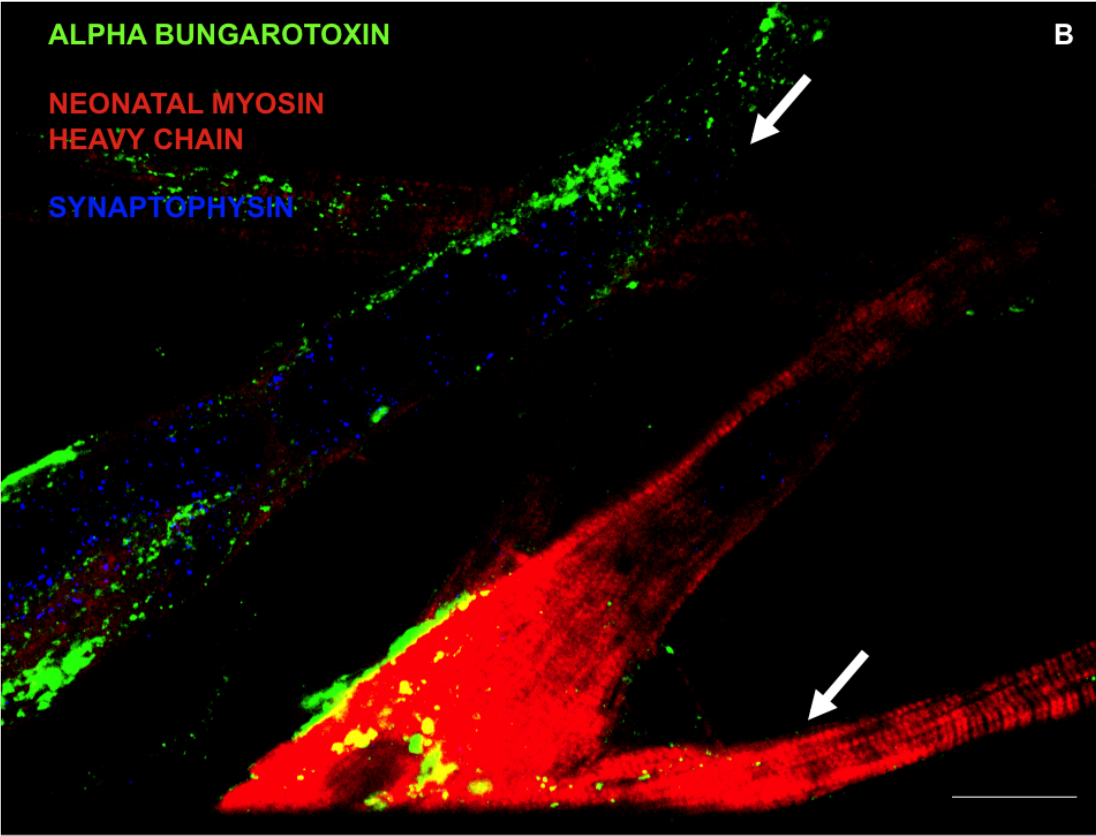
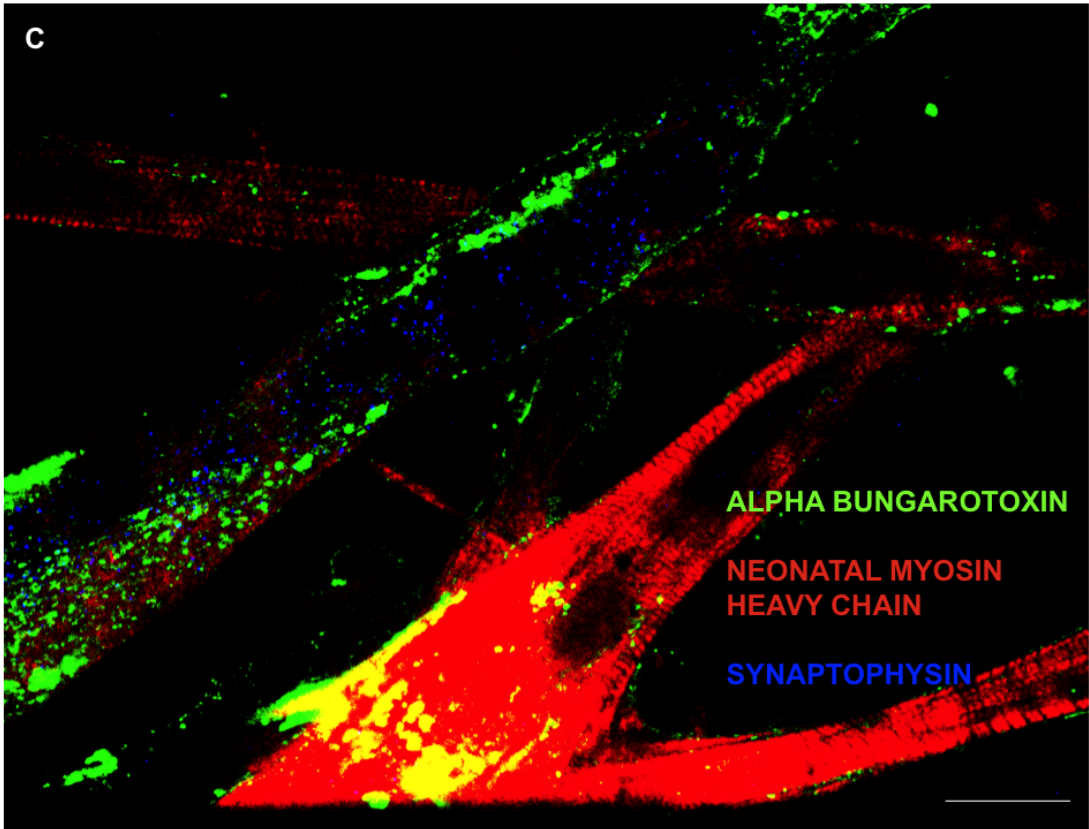


Figure 39. A, B: No NMJs were observed in these striated muscle. Although alpha-bungarotoxin was observed on the surface of the myotubes indicating the clustering of the acetylcholine receptors. But no distinct colocalization of synaptophysin was observed in close proximity of acetylcholine receptor clusters. A. The phase picture of the myotube. B. Immuno-stained picture of the same myotube with alpha-bungarotoxin, N3.36 and synaptophysin. The scale bar for both the pictures is 50 μ .

In figure 40 (A-D), we showed that NMJ formation took place on a myotube in culture which did not stained for neonatal myosin heavy chain.







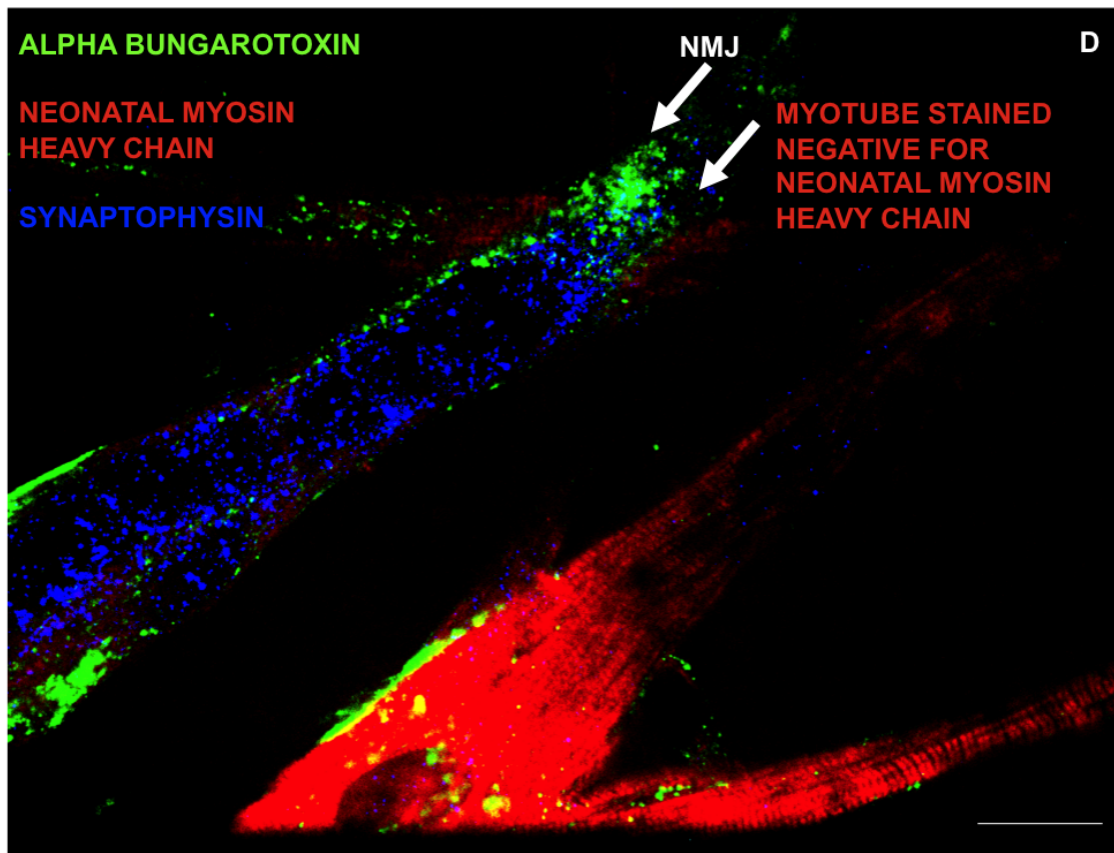


Figure 40. Formation of NMJ on a myotube which was not positive for N3.36. the cultures was stained with alpha-bungarotoxin, N3.36 and synaptophysin. A. Phase picture showing the different morphologies of myotubes in the co-culture. B-D. NMJ formation was observed on a myotube which was not positive for N3.36. Possibly the myotube on which NMJ was formed was still immature and did not express the neonatal myosin heavy chain (N3.36).

Previously, we developed the first defined system model to co-culture embryonic motoneuron and fetal skeletal muscle[5]. Our previous model was not suitable for long-term tissue engineering studies and the muscle in the culture only expressed the early muscle marker, fetal MHC. In this study, we have documented significant improvement over our previous motoneuron-skeletal muscle co-culture model system. This new improved culture model supported long-term co-culture of the motoneuron and muscle, resulted in more adult like morphology of the muscle and exhibited higher density of the neuromuscular junction (NMJ) formation. Our findings were supported by morphological and immunocytochemical evidence.

We have improved the medium composition. This new, improved medium consisted of 12 additional growth factors which promote motoneuron, skeletal muscle and neuromuscular junction differentiation. Additionally, we have made certain modification in the culture methodology (Figure 7.1). We have introduced the use of a specialized medium called NB4Activ for maintaining the cultures. In our previous model we had used Neurobasal/B27 as the changing medium. NB4Activ medium was developed recently for culturing brain neurons by Gregory J. Brewer in order to replace Neurobasal/B27. NB4Activ improved the electrical properties of the hippocampal neurons in the culture[110]. We started to use this medium for in order to enhance the survival of the motoneurons in pure culture. Interestingly, when we introduced this medium in our co-culture system, we observed long-term motoneuron and muscle survival and enhanced development of muscle. Specifically, 90% of the myotubes exhibited expression of a more mature marker of the

skeletal muscle, neonatal MHC (N3.36). In our previous motoneuron-skeletal muscle co-culture model, we did not observe the expression of neonatal MHC proteins in the myotubes[5]. Interestingly when this same medium and the same protocol was used to culture pure skeletal muscle we observed certain striking differences. The pure muscle culture survived longer, exhibited characteristic striations, but only a very small percentage of myotubes expressed N3.36 (unpublished data). Current research suggests, the N3.36 expression in skeletal muscle in the culture is either influenced by the motoneurons or by certain factors secreted by the motoneurons. The MHC class switching occurring in this system has been influenced by the presence of the Nb4Activ base medium and the additionally growth factors. This needs further studies in order to dissect the specific factors responsible for the activation of the molecular pathways regulating N3.36 expression in pure skeletal muscle culture and in the skeletal muscle-motoneuron co-culture.

As shown in Figure 38 and 40 this newly developed system resulted in enhanced NMJ development at later days due to extended life of the culture. The development of additional NMJs is likely a combination of both the enhanced medium formulation and the increased culture longevity. These improvements facilitate the study of NMJ maintenance, a process known to be influenced by Schwann cells. The improved understanding of the factors or cell-cell interactions required for NMJ maintenance would be a significant finding.

In summary, this work successfully demonstrated the development of a defined, novel bio-hybrid technology to tissue engineer neuromuscular junctions in the presence of a chemically defined medium and a synthetic silane substrate. This model system provides a better understanding of the minimal growth factor and substrate interactions necessary for NMJ formation. It also provides a basic system that in the future can be utilized for nerve-muscle and stretch reflex arc investigations, regenerative medicine and development of limb prosthetic devices.

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Part 3: Tissue Engineering Adult Mammalian Spinal Cord

CHAPTER 8: ADULT RAT SPINAL CORD CULTURE ON AN ORGANOSILANE SURFACE IN A NOVEL SERUM-FREE MEDIUM. IN VITRO CELL DEV BIOL ANIM. 2005 NOV-DEC;41(10):343-8.)

Introduction

Culture models have only been able to study the spinal cord regeneration of inframammalian vertebrates [1], amphibians [2] and achieved limited success in mammalian systems[3-5]. Recently, Brewer [6, 7] has cultured adult mammalian cortical and hippocampal neurons and shown that these adult central nervous system neurons are capable of survival and proliferation *in vitro*. However, a robust cell culture model of adult spinal cord neurons has remained elusive, until now. Previously, we have successfully created an *in vitro* defined model system for studying embryonic rat spinal cord motoneurons [8]. Here, we have advanced the scope of our previously developed model in order to culture adult rat spinal cord neurons.

This study documents the development of a defined *in vitro* culture system that promotes the regeneration and growth of dissociated adult rat spinal cord neurons. This culture system is comprised of a patternable [9], non-biological, cell growth promoting organosilane substrate, N-1[3-(trimethoxysilyl)propyl]-diethylenetriamine (DETA) coated on glass surface [8-14] and an empirically derived novel serum-free medium, supplemented with specific growth factors. We show the feasibility of using this synthetic silane substrate, combined with a novel serum-free medium, to create a long-term cell culture model from the dissociated cells of adult rat spinal cord. This culture

system will be a useful tool to study adult mammalian spinal neuron patterning, repair, myelination, degeneration and to screen different putative drug candidates for spinal cord repair and degenerative diseases of the spinal cord such as multiple sclerosis and amyotrophic lateral sclerosis (ALS).

Methods and Materials

Surface modification

Glass coverslips (Thomas Scientific 6661F52, 22×22 mm² No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 min at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904) according to Ravenscroft [9]. The DETA coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried (Figure 41 a).

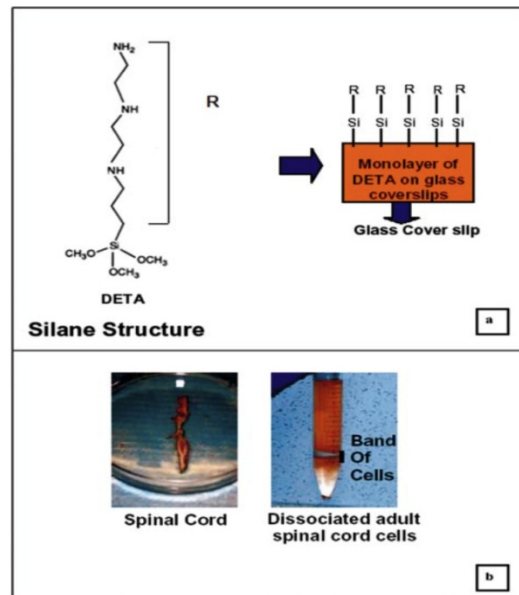


Figure 41. (a) Structure of a N-1(3-[trimethoxysilyl]propyl)-diethylenetriamine (DETA) molecule. Cartoon showing the DETA coating on a glass coverslip. (b) Isolated fragment of adult rat spinal cord (left). Major band of spinal cord cells obtained after optiprep gradient centrifugation (right).

Surface characterization

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray Photoelectron Spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1 s and C1 s scans, utilizing monochromatic Al K α excitation, were obtained.

Isolation and culture of rat spinal cord

Spinal cords were isolated from adult rats and the meninges were removed from the spinal cord. The spinal cord was then cut into small pieces and collected in cold Hibernate A, glutamine (0.5mM) and B27. Next, the tissue was enzymatically digested for 30 minutes in papain (2mg/ml). The tissue was triturated in 6 ml of fresh Hibernate A (www.BrainBits.com), glutamine (0.5mM) and B27 (Invitrogen). The 6 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate A/glutamine 0.5mM/B27) and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate A/ glutamine 0.5mM/B27 followed by centrifugation for 15 min, using 800g, at 4°C. The top 7 ml of the supernatant was aspirated. The next 2.75 ml from the major band and below was collected and diluted in 5ml Hibernate A/B27 and centrifuged at 600 g for 2 minutes (Figure 8.1.b). The pellet was resuspended in Hibernate A/B27, and after a second centrifugation, the pellet was resuspended in the culture medium (see Table 8.1 for the specific composition). Three-fourths of the culture medium was changed during the first 2-3 days in culture and thereafter half of the medium was changed after every three days [6, 7].

Table 10: Composition of 500 ml serum-free medium

Component	Source	Catalogue #	Amount
Neurobasal A	Invitrogen	11415064	500 ml
Gluta Max (100X)	Invitrogen	35050-061	5ml
B27 Supplement (50x)	Invitrogen	17504044	10 ml
Acidic Fibroblast Growth Factor (aFGF)	Invitrogen	13256029	10ng/ml
Brain Derived Neurotrophic Factor (BDNF)	Invitrogen	10908019	10ng/ml
Glial Derived Neurotrophic Factor (GDNF)	Invitrogen	10907012	10ng/ml
Neurotrophin 3 (NT-3)	Sigma	N1905	5ng/ml
Cardiotrophin-1 (CT-1)	Cell Sciences	CRC700B	10ng/ml
Vitronectin	Sigma	V0132	100ng/ml
Heparin Sulphate	Sigma	D9808	10ng/ml
Antibiotic-Antimycotic (100X)	Invitrogen	15240-062	5ml
pH Osmolarity	7.3 and 320 milliOsm		

Immunocytochemistry

In preparation for staining with anti-neurofilament 150 and anti-GFAP antibodies the coverslips were rinsed free of medium with PBS and fixed for 20 min at room temperature with 10% glacial acetic acid and 90% ethanol. The staining of coverslips using anti-neuron specific enolase and anti-Islet antibody 4D5 were similarly rinsed free of medium with PBS but fixed with 4% paraformaldehyde in PBS for 20 min. After rinsing twice with PBS, cells were permeabilized for 5 min with 0.5% Triton X-100 in PBS. After rinsing with PBS, the non-specific sites were blocked and cells permeabilized with 5% normal donkey serum and 0.5% Triton X-100 in PBS. Cells were incubated overnight at 4°C with rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:100) and mouse anti-GFAP monoclonal antibody (Chemicon MAB360, diluted 1:400), mouse anti neuron-specific enolase gamma-gamma monoclonal antibody (Chemicon, MAB 314, diluted 1:10) and Anti-Islet antibody 4D5 [15] (Developmental Studies Hybridoma Bank, diluted 1:50), in the blocking solution. After overnight incubation, the coverslips were rinsed four times with PBS and then incubated with the appropriate secondary antibodies for 2h. After rinsing four times in PBS, the cover slips were mounted with Vectashield mounting medium (H1000, Vector Laboratories) onto slides. The coverslips were visualized and photographed using a Zeiss LSM 510 Confocal microscope. Controls without primary antibody were negative.

Electrophysiology

Whole-cell patch clamp recordings were performed in a recording chamber placed on the stage of a Zeiss Axioscope 2 FS Plus upright microscope in Neurobasal culture medium (pH was adjusted to 7.3 with HEPES) at room temperature. Patch pipettes (6-8 Mohm) were filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, MgCl₂ 2, Na₂ATP 5, HEPES 10; pH = 7.2). Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon, Union City, CA) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis was performed with pClamp 8 (Axon) software. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -70 mV holding potential. Whole cell capacitance and series resistance was compensated and a p/6 protocol was used. The access resistance was less than 22 Mohm. Action potentials were measured with 1 s depolarizing current injections from the -70 mV holding potential [8].

Result and Discussion

Surface modification

Static contact angle and XPS analysis were used for the validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles ($40.64^\circ \pm 2.9/\text{mean} \pm \text{SD}$) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results [8-14]. Based on the ratio of the N (401 and 399 eV) and the Si $2p_{3/2}$ peaks, XPS measurements indicated that a complete monolayer of DETA was formed on the coverslips [8-14] (Figure 42).

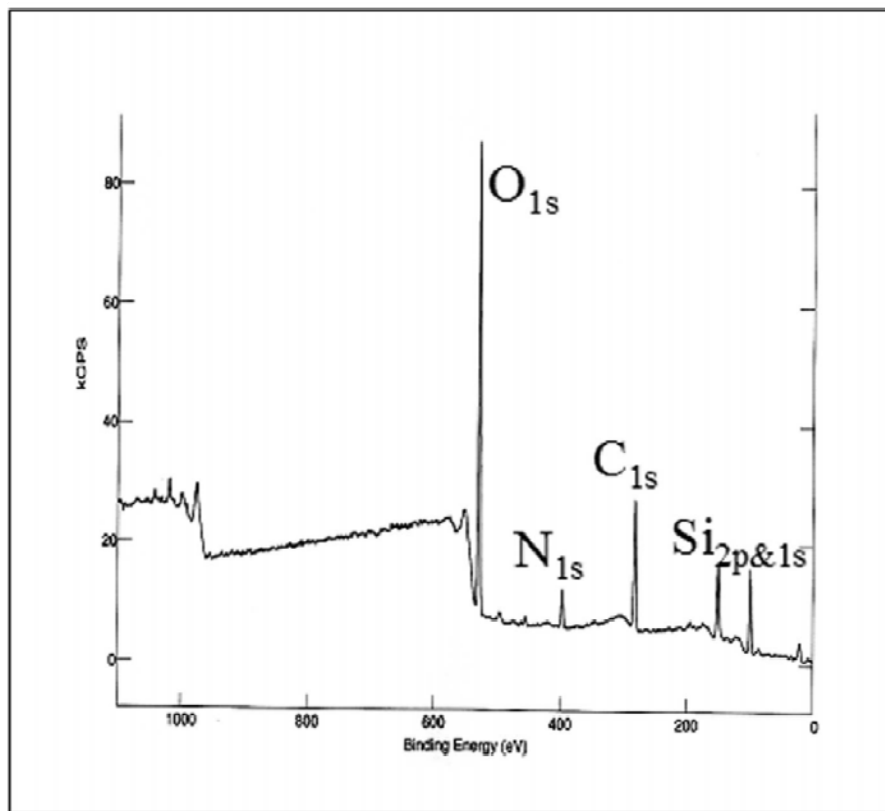


Figure 42. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, CAM 200) (data not shown) and by X-ray photoelectron spectroscopy (XPS; Kratos Axis 165) by monitoring the N 1 s peak. This figure shows an XPS survey scan of the N-1(3-[trimethoxysilyl]propyl)-diethylenetriamine monolayer.

Adult spinal cord culture and immunocytochemistry

Dissociated cells from normal adult rat spinal cord were grown for periods of 3 to 5 weeks on DETA in the presence of a serum-free defined medium (Table 10). The neurons began to send out processes by day 6 (± 3) (Figure 43 a) and indicated extensive neurite outgrowth by day 18 (± 3) in culture ($N > 80$, where $N = \#$ of rats) (Figure 43 b). The double staining of the culture with anti-neurofilament and anti GFAP antibodies on day 25 of the experiment showed that the culture contained a mixture of neuronal (73% (SD = 4.0), $n = 6$, where $n = \#$ of coverslips) and glial (27% (SD = 4.5), $n = 6$) cells (Figure 43 c). The neurons were further characterized by staining with anti-neuron specific enolase (Figure 43 d) and anti-Islet-1 antibodies (Figure 43 e). A smaller fraction of neurons (10% (SD = 3.5), $n = 7$) stained for anti-Islet-1 antibody, a specific marker for motoneurons [15].

Electrophysiology

Whole cell patch clamp experiments were performed on 10 day old cultures. About 30% of the recorded cells expressed voltage dependent sodium and potassium currents (Figure 8.3.f) and generated single action potentials (data not shown).

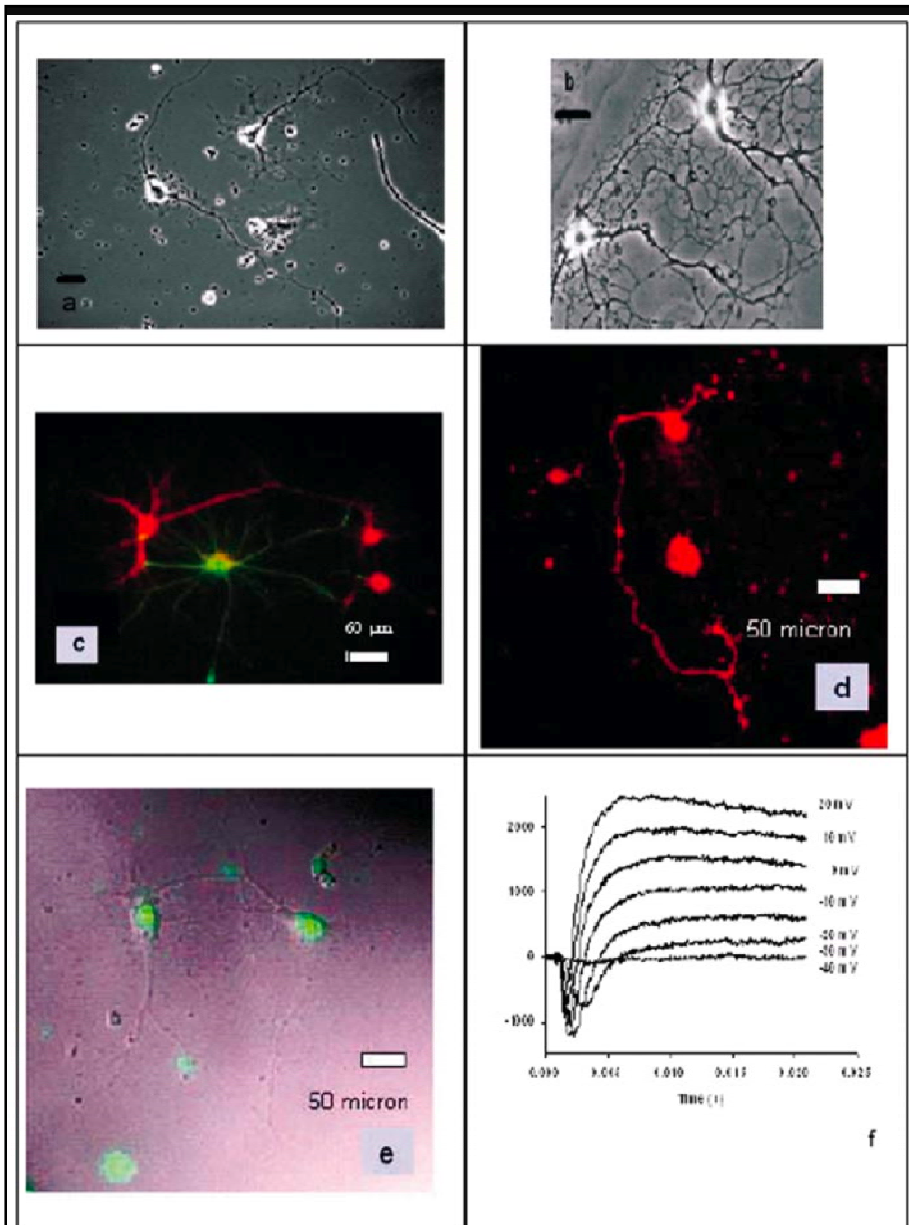


Figure 43. a. Phase contrast picture of neuronal and glial cells in the adult spinal cord culture (day 6 *in vitro*), Scale bar: 50 micron. b. Phase contrast picture of neuronal and glial cells in the adult spinal cord culture (day 25 *in vitro*), Scale bar: 50 micron. c. Immunostaining with anti-neurofilament 150, a neuron specific marker (red) and anti-GFAP, a glial cell marker (green) (day 25 day *in vitro*). d. Second neuronal specific marker for anti-NSE (red) (day 15 *in vitro*). e. Anti-Islet-1 staining of cells that exhibited a neuronal morphology (day 25 *in vitro*). The nucleus is brightly stained with Islet-1 (green) which is a putative motoneuron marker. f. Representative voltage clamp recordings obtained from neuronal cells on day 10 *in vitro*. Voltage dependent ionic currents were evoked by voltage steps from -40 to $+20$ mV.

Use of an Engineered Synthetic Substrate: DETA

The engineered growth substrate consisted of a glass surface coated with a DETA self-assembled monolayer, which had previously been shown to support neuronal, endothelial and cardiac cell growth [8-14] and had also been used in creating high resolution, *in vitro* patterned circuits of embryonic hippocampal neurons [9]. There are three major rational for using the synthetic DETA substrate in this study.

First, the DETA substrate can be subsequently patterned at a high resolution to study engineered *in vitro* spinal cord neuron networks, which is difficult to achieve with regular biological substrates like laminin [8]. Previously, the creation of such engineered networks was shown to be feasible with embryonic hippocampal neurons [9] and embryonic rat motoneurons (unpublished data). Currently, we are working on developing a patterned network of adult spinal cord neurons in order to understand the complex information processing taking place at the level of the spinal cord.

Secondly, DETA substrate can be coupled with specific extracellular matrix molecules [16], and different contact signaling molecules, in order to systematically study the specific role of such molecules in remyelination, neurodegeneration and axonal growth inhibition during spinal cord injury and recovery [17].

Finally, high resolution patterned DETA substrates have been shown to promote guided axonal growth and direct axonal and dendritic process extension at the level of a single neuron [9, 18]. In the future, such surface modification techniques could be used as a powerful tool to create a neuro-electric interface chip in order to bridge the injured fragment of the spinal cord [19-23].

Cell Culture

The spinal cord culture techniques followed in this study were similar to the techniques developed by Brewer [6, 7] in order to culture adult rat hippocampal and cortical neurons. Currently, we have a mixed culture of neuronal and glial cells. We are using different cell separation techniques in order to isolate different cell types of the spinal cord and study their respective physiology. One of the challenging issues in such an *in vitro* cell culture model is to reduce the amount of cellular debris during cell plating. The cellular debris also contains several growth inhibitory molecules which can result in a slow recovery of the surviving neurons [24-29]. During the initial ten days of culture we observed very limited growth, but this improved with time. As half of the medium was changed every 3-4 days, the inhibitory cellular debris could have been slowly washing away and, in support of this hypothesis, after day 18 we observed extensive neurite outgrowth from most of the neurons. The growth continued for the next 3-4 weeks. In our future studies we will develop different techniques to reduce the initial plating debris so as to promote faster regeneration.

Development of the Serum-Free Medium

The serum-free medium has been developed based on our previous results and published results from others outlined below. The defined serum-free medium consists of Neurobasal A, B27 supplement [6, 7], a-FGF [30-33] (10ng/ml), heparin sulphate [30] (10ng/ml), neurotrophin-3 (NT-3) [34-38] (5ng/ml), glial derived neurotrophic factor (GDNF) [29, 39] (10ng/ml), brain derived neurotrophic factor (BDNF) [29, 34] (10ng/ml), cardiotrophin-1 (CT-1) [29, 40, 41] (10ng/ml) and vitronectin (100ng/ml). The rationale for selecting these growth factors is based on the distribution of their receptors in the CNS and their therapeutic role in mammalian adult and embryonic spinal cord regeneration [34, 35, 37, 38, 42-44].

We, and others, had shown previously [8, 45, 46] that embryonic spinal motoneurons can be grown in a defined system using neurobasal medium, B27 supplement, GDNF (1ng/ml), BDNF (1ng/ml) and CT-1 (10ng/ml). In this study we have added four additional factors: a-FGF [30-33], heparin sulphate [30], NT-3 [34-38], vitronectin, and have now demonstrated long term survival and growth of adult spinal cord neurons in a defined media using this new formulation.

Analysis of the distribution of a-FGF in the adult rat nervous system indicated that a-FGF-like bioactivity was very high in the periphery and unevenly distributed in the CNS, with the highest a-FGF levels being observed in spinal cord [30]. Further, a strong immunohistochemical localization of a-FGF is found in all adult motoneurons. No staining for a-FGF was observed for non-neuronal cells [30]. We believe that a-FGF leaking from an injured motoneuron may be involved in initiating

repair responses in the motoneuron in an autocrine manner, as previously proposed by Eckenstein et al [30]. Motoneuron survival in vivo can be supported by FGFs. Eckenstein has also shown that a-FGF requires exogenous heparin [30]. This led to the addition of heparin sulphate along with a-FGF to the medium.

Hasse et al [36] had demonstrated that adenovirus-mediated gene transfer of NT-3 can produce substantial therapeutic effects in mouse mutant PMN (progressive motor neuronopathy). We had also observed that NT-3, along with vitronectin, improved the health of the embryonic motoneuron cultures and was the reason NT-3 and vitronectin were used. Similar neuroprotective effects had been observed in adult injured motoneurons by the adenoviral gene transfer of GDNF and BDNF [29]. Recently, the therapeutic effect of in vivo electrotransfer of the CT-1 gene into skeletal muscle had demonstrated the slowing down of motor neuron degeneration in PMN mice [41]. These recent findings led us to add GDNF, BDNF and CT-1 to the medium.

One of the major components of the B27 supplement [6, 7] is retinyl acetate, an analog of pro-retinoic acid. Retinoic acid and its receptor, beta2, has been shown to promote neurite outgrowth in the adult mouse spinal cord in vitro [47, 48]. We believe that the presence of retinyl acetate in the B27 supplement has further accelerated the regeneration process by activating retinoic acid receptors. One future line of work is to understand the role of individual growth factors and to further refine the medium for studying remyelination of spinal neurons after injury.

Electrophysiology

Preliminary electrophysiology experiments indicated that 30% of the recorded cells expressed voltage dependent sodium and potassium currents and were able to generate single action potentials. Previously, similar single action potentials were observed in mitogen expanded neural precursor cells of adult rat spinal cord by Liu et al [49]. Further electrophysiological characterizations are being undertaken to determine synaptic connectivity events between motoneurons and their targets.

These are the first studies to demonstrate that adult rat spinal cord cells can be cultured in a completely defined serum-free medium and on a synthetic silane substrate. This in vitro culture system will be a useful tool to study adult mammalian spinal neuron repair, myelination, degeneration, as well as to screen different novel and putative drug candidates for spinal cord repair and degenerative diseases of spinal cord.

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CHAPTER 9: AUTO-CATALYTIC CERIA NANOPARTICLES OFFER NEUROPROTECTION TO ADULT RAT SPINAL CORD NEURONS. BIOMATERIALS. 2007 APR;28(10):1918-25

Introduction

In this study we have demonstrated that auto-catalytic nano-Ceria particles will enhance survival of adult spinal cord neurons in a unique, defined in vitro system. Ultra fine, non-agglomerated Cerium oxide nanoparticles (2-5 nm) were synthesized by a microemulsion process [1] for their suitability for neuroprotective applications in spinal cord injury and degenerative diseases of the central nervous system (CNS). To test the general biocompatibility and neuroprotection capability of the synthesized nanoparticles, we evaluated the activity of nano-Ceria in a novel serum-free cell culture model of adult rat spinal cord [2]. Administration of a single dose of nano-Ceria (10 nM) at the time of cell plating promotes significantly higher neuronal survival as compared to control cultures. Nanoparticle treated neurons indicated normal electrical activity as compared to the control culture to demonstrate functional biocompatibility. Nano-Ceria treated cells also had significantly higher cell survival upon hydrogen peroxide-induced oxidative injury in the adult spinal cord model system. Based on our cell culture assays, UV-visible spectroscopic studies, a hydrogen peroxide-induced oxidative injury assay and our proposed working hypothesis, we conclude that the supplementation of adult neuronal cultures with auto-catalytic Ceria nanoparticles has a significant effect in neuronal survival and retention of function.

Cerium oxide is a rare earth oxide that is found in the lanthanide series of the periodic table. It is used in various applications such as: histochemistry [3, 4] electrolytes for solid oxide fuel cells [5], ultraviolet absorbents [6], oxygen sensors [7, 8], and automotive catalytic converters [9]. Nanocrystalline Cerium oxide exhibits a blue shift in the ultra violet absorption spectrum [6], the shifting and broadening of Raman allowed modes [10] and lattice expansion as compared to bulk cerium oxide [6, 11]. It was these attributes of this material that were an early indicator that it has unique properties.

These results demonstrate that the use of nano-Ceria could prove beneficial for the in vivo repair of spinal cord neurons based on our experiments evaluating the nano-ceria in a more realistic in vitro model of spinal cord utilizing adult CNS cells. It is also anticipated that they could be good candidates for drug delivery and imaging applications. Based on the surface chemical properties of Ceria nanoparticles [12] we propose a hypothesis to explain the neuroprotective role of this material.

Methods and Materials

Nanoparticle Synthesis

Cerium oxide nanoparticles were prepared by a microemulsion method. The nanosized micelles act as reactors for particle formation. The microemulsion system consisted of the surfactant, sodium bis(2-ethylhexyl) sulphosuccinate (AOT), toluene and water. All the chemicals were purchased from Aldrich Chemicals Company, Inc. Details of the synthesis are published elsewhere [1]. The particles obtained in toluene were re-dispersed in water by evaporating the toluene prior to use in the cell culture studies.

Nanoparticles Characterization

The particle morphology was studied using HRTEM. The surface chemistry of the nano-Ceria particles was studied using XPS. The Ceria nanoparticles were deposited on the carbon coated copper grid for HRTEM analysis by the dip coating method. The HRTEM images of the as prepared particles were obtained with a Philips (Tecnai Series) transmission electron microscope operating at 300 keV. The XPS data was obtained using a 5400 PHI ESCA (XPS) spectrometer. The base pressure during XPS analysis was 10^{-9} Torr and Mg- K_{α} X-ray radiation (1253.6eV) at a power of 200 watts was used. The binding energy of Au ($4f_{7/2}$) at 84.0 ± 0.1 eV was used to calibrate the binding energy scale of the spectrometer. Any charging shift produced in the spectrum

was corrected by referencing to the C (1s) position (284.6 eV) [[13]. XPS spectra smoothing and baseline subtraction was carried out using PeakFit (Version 4) software.

Cell Culture

Surface modification of the coverslips for cell culture: The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned glass surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. The detailed procedure is described elsewhere [14, 15]

Surface characterization of the coverslips: Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by XPS (Fisions 220i). XPS survey scans, as well as high-resolution N 1s and C 1s scans, utilizing monochromatic Al K α excitation, were obtained according to our previously published procedures [2, 14-17].

Isolation and culture of rat spinal cord: Spinal cords were isolated from euthanized adult rats (average age was 3-4 months) and the meninges were removed from the spinal cord. One single spinal cord from an adult rat weighs 1.10 g (+/- 0.05). The harvested spinal cord was cut into small

pieces and collected in cold Hibernate A [[18] (www.BrainBits.com), GlutaMAX™, an antibiotic-antimycotic and B27 (Invitrogen). Next, the tissue was enzymatically digested for 30 minutes in papain (2mg/ml). The tissue was dissociated in 6 ml of fresh Hibernate A, GlutaMAX™, an antibiotic-antimycotic and B27. The 6 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate A/ GlutaMAX™ / antibiotic-antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27) followed by centrifugation for 15 min, using 800g, at 4°C. The top 7 ml of the supernatant was aspirated. The next 2.75 ml from the major band and below was collected and diluted in 5ml Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27 and centrifuged at 600 g for 2 minutes. The pellet was resuspended in Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27, and after a second centrifugation, the pellet was resuspended in the culture medium. Approximately 12,000-13,000 live cells are harvested from one adult rat spinal cord. 1000 cells were plated on each coverslip (22 X 22 mm²) at 2 cells/mm². The culture medium was changed completely after the first 2 days in culture and thereafter half of the medium was changed every four days [2, 19]. A molecular Probe's L-3224 Live/Dead Assay kit was used for the live-dead assays [16].

Immunocytochemistry for quantification of neuronal and glial cells in control and nanoparticle treated culture: Rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:100) and mouse anti-GFAP monoclonal antibody (Chemicon MAB360, diluted 1:400),

were used for staining the neuronal and glial cells. The method for immunostaining is described in detail elsewhere [2].

Electrophysiology of nanoparticle treated culture: Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon, Union City, CA) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis was performed with pClamp 8 (Axon) software. The detailed protocols are documented in our previous work [2, 16].

Results and Discussion

A microemulsion process was used to synthesize the cerium oxide nanoparticles (Figure 44.a) and they were characterized for morphology and surface chemistry by high-resolution transmission electron microscopy (HRTEM) and X-ray photoelectron spectroscopy (XPS). HRTEM indicated the formation of uniformly distributed, non-agglomerated nanoparticles of Cerium oxide in the range of 3-5nm as shown in the image in Figure 44.1b. Figure 44.c shows a XPS spectrum that indicates a mixed valence state (Ce^{3+} and Ce^{4+}) for the synthesized Cerium oxide nanoparticles. These results are similar to our previously published results [1, 12, 20].

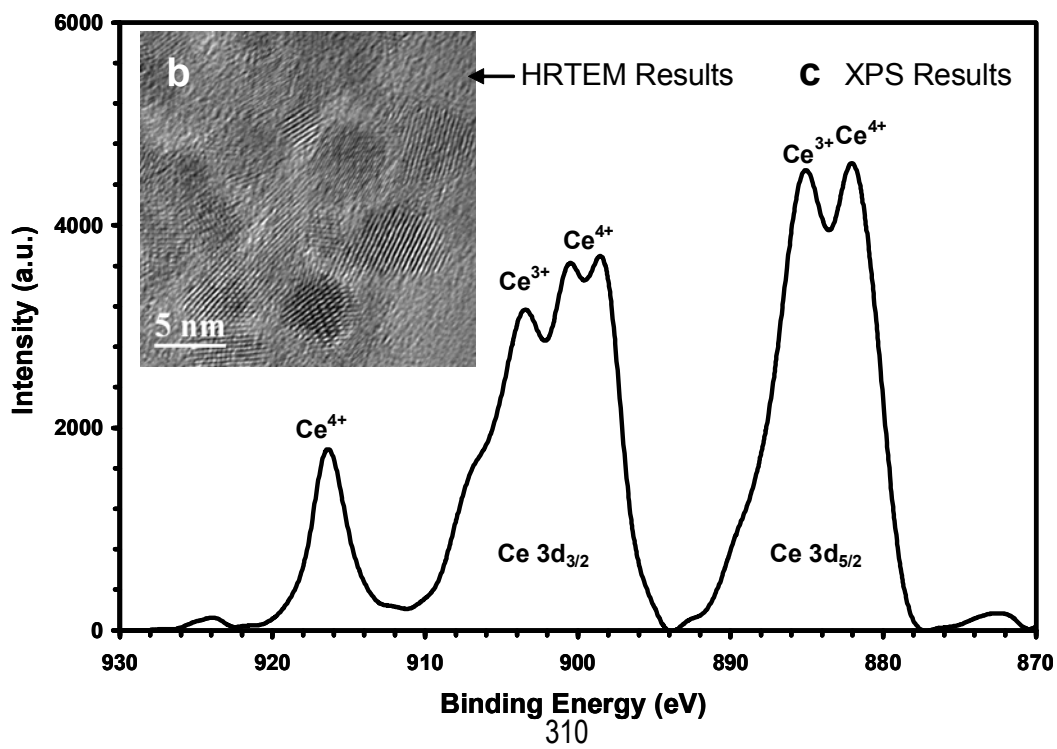
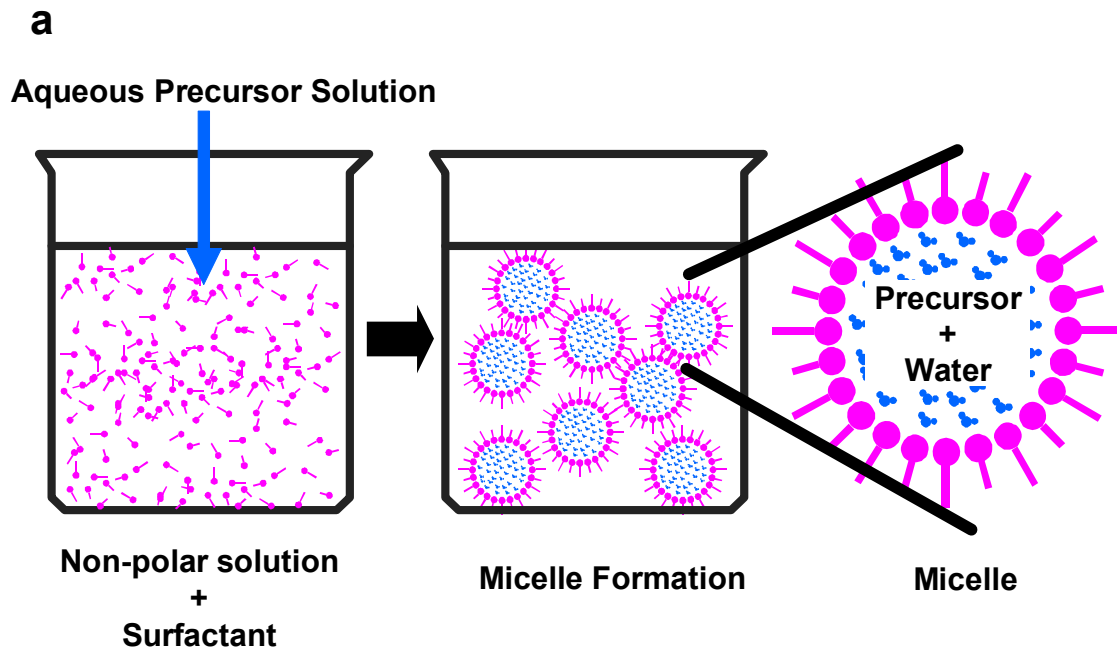


Figure 44. Synthesis and Characterization of Cerium oxide Nanoparticles. (a) Outline of the microemulsion technique to synthesize the nano-Ceria (b) XPS analysis of synthesized Cerium oxide nanoparticles showing the presence of both Ce³⁺ and Ce⁴⁺ valence states (c) HRTEM image of the synthesized nanoparticles showing uniform particle size in the range of 3-5 nm.

The in vitro studies with the synthesized nanoparticles were carried out in a serum-free cell culture model of adult rat spinal cord (Figure 45) which has been shown to promote growth and long-term survival of dissociated adult rat spinal cord neurons [2]. This system consists of a patternable [15], non-biological, cell growth promoting organosilane substrate, N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine (DETA)[2, 16, 17, 21, 22], coated on a glass surface combined with an empirically derived, novel serum-free medium and a reproducible cellular isolation and pre-plating methodology. The serum-free medium consisted of neurobasal A supplemented with B27 [23], GlutaMAX™, acidic fibroblast growth factor, heparin sulphate, neurotrophin-3, neurotrophin-4, ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor, glial derived neurotrophic factor, cardiotrophin-1, vitronectin and an antibiotic-antimycotic (Figure 45.b). The quality of the surface modified coverslips used for cell culture was monitored using static contact angle measurements and XPS analysis as previously described [2, 14, 16, 17]. Stable contact angles ($40.64^\circ \pm 2.9/\text{mean} \pm \text{SD}$) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results [2, 14, 16, 17, 24]. Based on the ratio of the N 1s (401 and 399 eV) and the Si 2p_{3/2} peaks, XPS measurements indicated that a monolayer of DETA (Figure 45.c) was formed on the coverslips [2, 14, 16, 17]. The cell isolation process from dissected adult rat spinal cord is briefly described in the methods.

The outline of the cell isolation and cell plating is shown in Figure 45.a and documented in detail in our previous work [2]. In each experiment, an equal volume of the cell suspension (1000 live cells

at a density of 2 cells/mm²) was plated on each coverslip. Of the total number of coverslips plated with cells, half of the coverslips were used for control cultures and the other half received a single dose of 10nM nano-Ceria at the time of cell plating. At two different time intervals, day 15 and day 30, live-dead assays and neuron-glia immunostaining assays were conducted to quantify cell viability and the surviving cell types in both the control and nano-Ceria treated cultures. A student's T-test was used for statistical analysis. The results are expressed as mean \pm SE, n = 6, where n stands for number of coverslips.

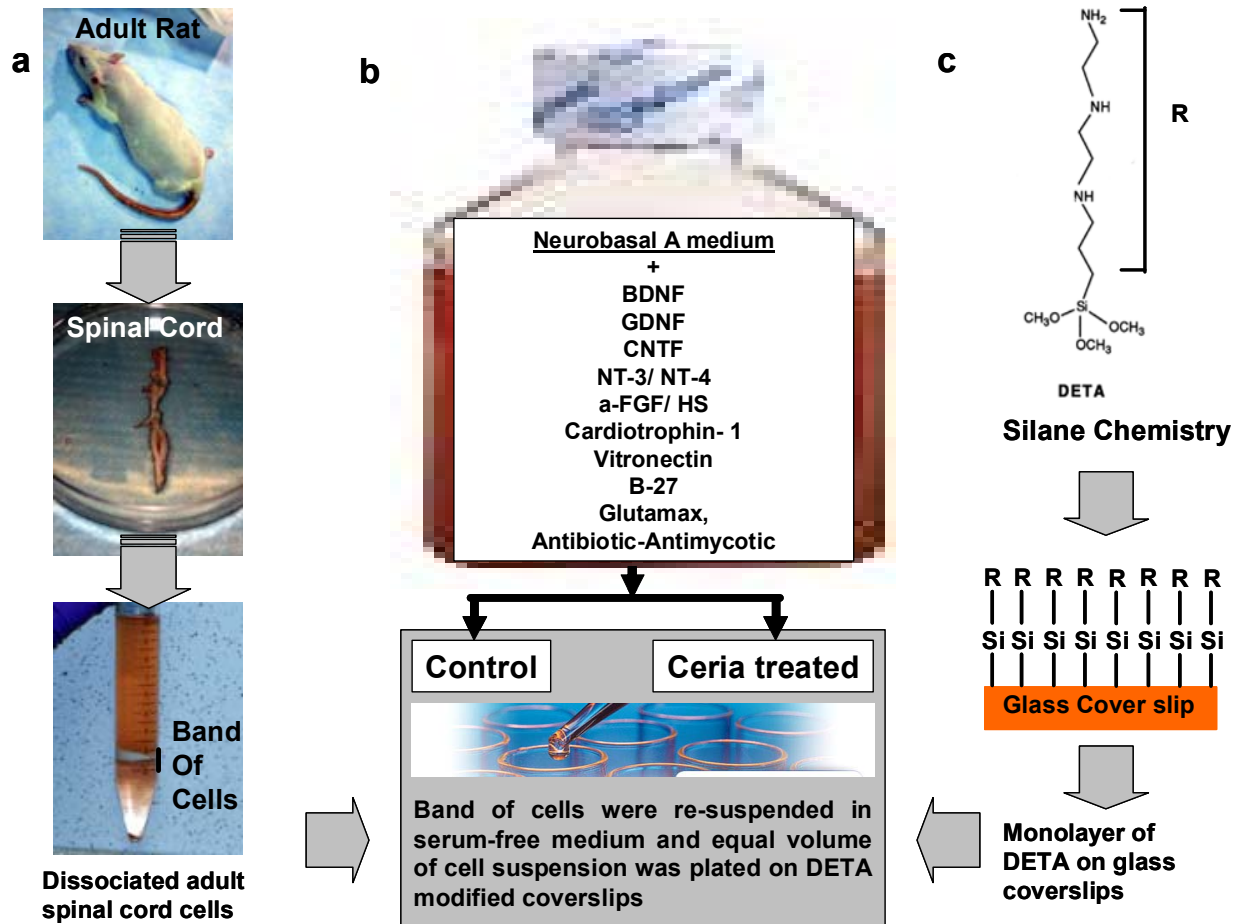


Figure 45. Adult Rat Spinal Cord Culture (a) Isolation of adult rat spinal cord cells from the whole cord (b) Development of serum-free culture medium using various growth factors (c) Surface modification of the glass cover slips for cell culture.

The total number of coverslips used for these assays were drawn from six different experiments. Live-dead cell assays (Figure 46.a) indicated a significantly higher cell survival at day 15 (617 ± 34 , $n = 6$) and at day 30 (472 ± 35 , $n = 6$) in nano-Ceria treated cultures as compared to the control cultures at day 15 (479 ± 37 , $n = 6$) and day 30 (328 ± 32 , $n = 6$). We also observed a significantly lower cell death at day 15 (59 ± 7 , $n = 6$) and day 30 (48 ± 7 , $n = 6$) in nano-Ceria treated cultures as compared to the control cultures on day 15 (110 ± 9 , $n = 6$) and day 30 (72 ± 8 , $n = 6$). Neurons and glial cells were identified by immunoreactivity for neurofilament 150 (neuronal marker) and glial fibrillary acidic protein (GFAP) (glial marker) antibodies respectively. The neuronal population was significantly higher in nano-Ceria treated cultures at day 15 (191 ± 40 , $n = 6$) and at day 30 (221 ± 12 , $n = 6$) compared to the control cultures on day 15 (71 ± 26 , $n = 6$) and day 30 (148 ± 9 , $n = 6$). There was no significant difference in glial cell population or populations of cells which stained for both neuron and glial markers in treated cultures compared to control cultures at either time interval (Figure 46.b).

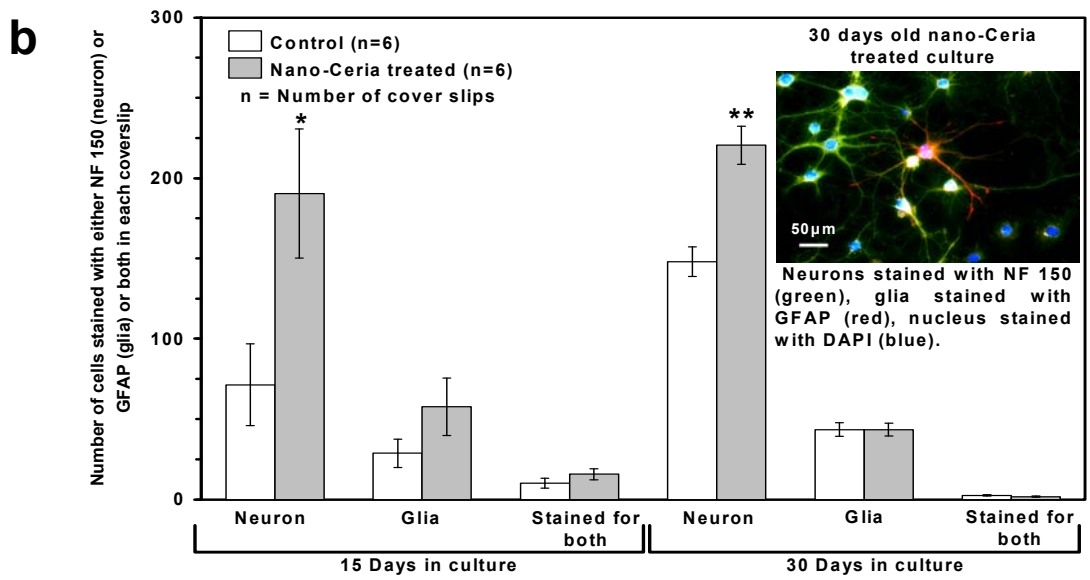
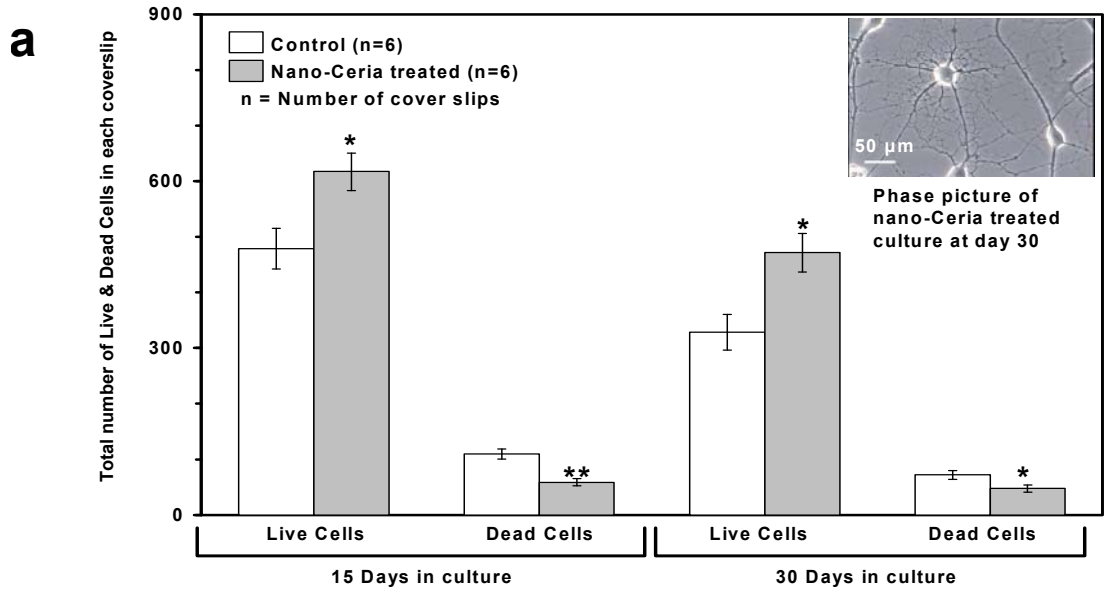


Figure 46. Live-Dead Assay, Neuron-Glial Cell Assay and Electrophysiological Studies of Control and nano-Ceria Treated Cultures of Adult Rat Spinal Cord (a) Live-dead cell assays indicated that nano-Ceria treated cultures had significantly higher cell survival and significantly less cell death at day 15 and day 30 in culture as compared to the control cultures (b) Neuron-glial cell assays indicated that a significantly high neuronal survival in treated cultures at day 15 and day 30 as compared to the control cultures. (Data are presented as Mean \pm SE, n = 6; * p < 0.05; ** p < 0.01).

Electrical activities of the nano-Ceria treated cultures were assessed using patch-clamp electrophysiology at day 30 in culture. The treated neurons expressed voltage dependent inward and outward currents (Figure 47.a) and generated single action potentials (Figure 47.b), similar to that observed for the controls and in other adult rat CNS cultures [2], [25].

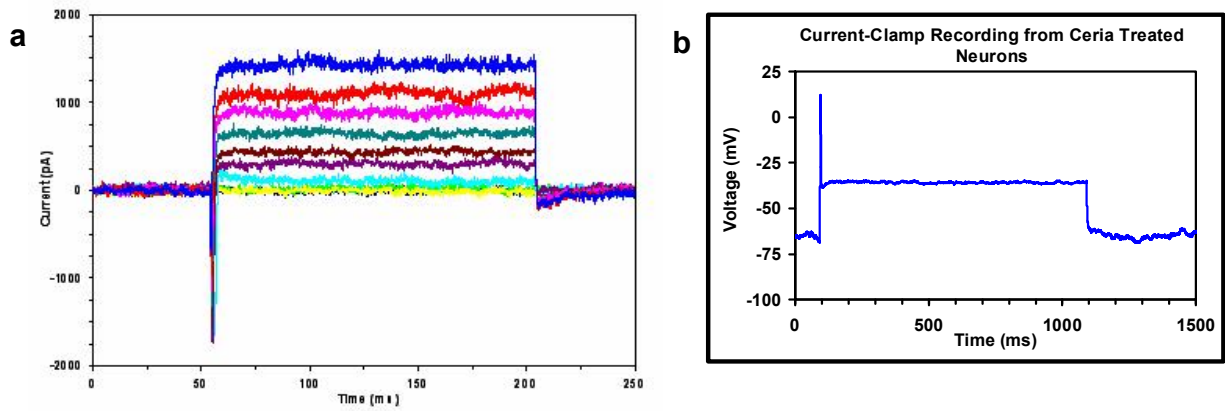
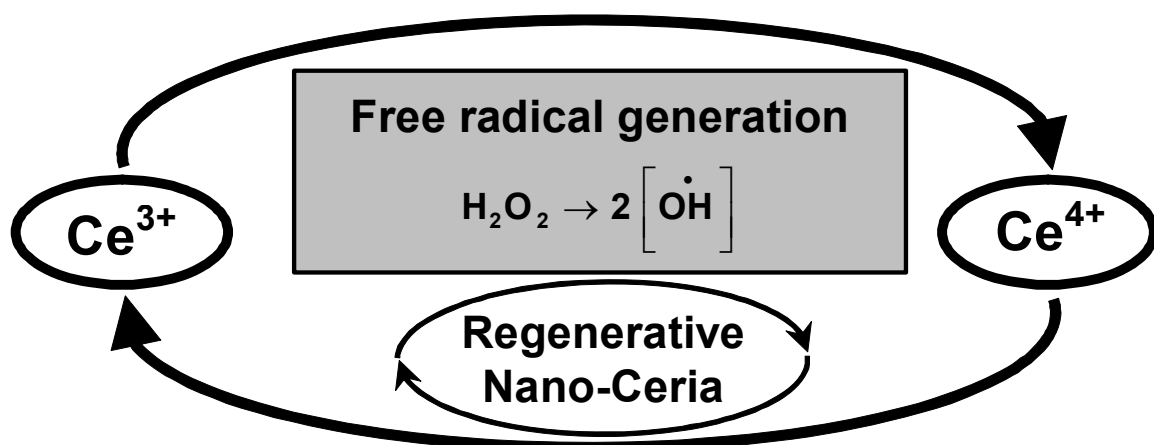
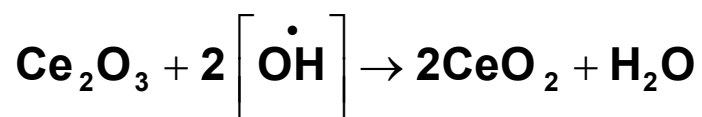


Figure 47. Voltage-clamp recording from a treated culture at day 30 (left) Current clamp recording indicating a single action potential in a nano-Ceria treated culture at day 30 (right).

We propose that the presence of the mixed valence states of Ce^{3+} and Ce^{4+} on the surface of the nano-Ceria act as an anti-oxidant that allow the nanoparticles to scavenge free radicals from the culture system. Another complex set of surface chemical reactions [26] between the ions in the cell culture medium and the nano-Ceria then appear to be involved in reversing the oxidation state from Ce^{4+} to Ce^{3+} . We believe that this is indicative of a cyclical regenerative, or auto-catalytic, reaction of the Ceria nanoparticles. The proposed mechanism is shown in Figure 48.

Free Radical Scavenging



Recovery by surface chemical reaction

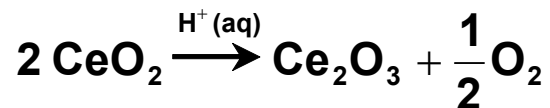


Figure 48. Schematic Detailing the Proposed Regenerative Properties of nano-Ceria and probable mechanism of Cerium oxide nanoparticles' free radical scavenging property and auto-catalytic behavior.

To demonstrate the auto-catalytic spectrum of a sample of the nano-Ceria solution was used as a control (black trace in the graph). We property of the engineered nano-Ceria particles, we carried out a UV-visible spectroscopic study of a nano-Ceria sol treated with 10mM hydrogen peroxide (Figure 49).

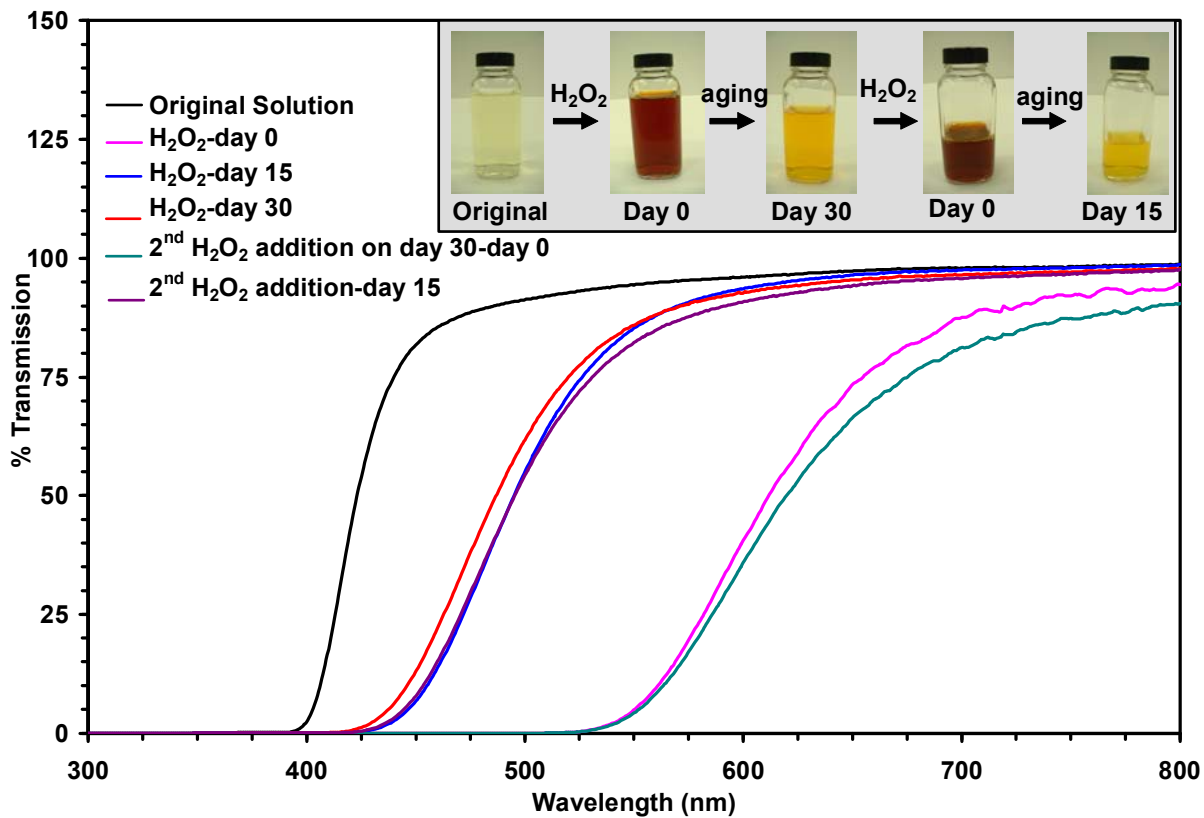


Figure 49. UV-visible study of Cerium Oxide nanoparticles treated with hydrogen peroxide at different time intervals.

In this reaction, hydrogen peroxide provides a source of hydroxyl radicals to mimic oxidative stress found in vivo. The UV-Visible added hydrogen peroxide to this solution and observed a shift in the spectrum to the right or to the lower energy portion of the spectrum (pink trace). This shift is postulated to be due to a change in the oxidation state [27] from Ce^{3+} to Ce^{4+} . The nano-Ceria sample treated with hydrogen peroxide was then kept in the dark for 30 days. UV-Visible spectra of these samples were then taken at day 15 and day 30 (blue and red traces for the day 15 and day 30 spectra, respectively). A gradual shift in the spectra to the left was seen over time. This gradual higher energy shift reflects the regeneration ($Ce^{4+} \rightarrow Ce^{3+}$) of the cerium oxide nanoparticles. When an additional hydrogen peroxide dose was administered to the solution on day 30, the UV-Visible spectrum again shifted to lower energy (green trace) which was followed by a gradual shift to the lower wavelength, as seen previously. The shift to a higher energy state on exposure to hydrogen peroxide with a recovery toward a lower energy state indicates that the nano-Ceria particles have the capacity for catalytic oxidative recovery ($Ce^{3+} \rightarrow Ce^{4+} \rightarrow Ce^{3+}$) This indicates that nano-ceria exhibits a mechanism in which the engineered particle provides a new material for life science applications with unprecedented antioxidant activity and pseudo-infinite half-life. The auto-regenerative anti-oxidant property of these nanoparticles appears to be the key to its neuroprotective action.

The auto-catalytic properties of the ceria oxide particles were further demonstrated in a hydrogen peroxide-induced oxidative injury model utilizing the adult spinal cord model system. A 100 mM

hydrogen peroxide solution was added for 1h to both a control culture and a nano-Ceria treated culture at day 30. After 1h of hydrogen peroxide treatment, the cell viability was assayed using a live-dead assay kit. The nano-Ceria treated cultures had a significantly higher number of live cells (82 ± 18 , $n = 6$) as compared to the control (29 ± 6 , $n = 6$). We did not observe any significant difference in the number of dead cells between nano-Ceria treated (362 ± 73 , $n = 6$) and control (309 ± 44 , $n = 6$) cultures after hydrogen peroxide treatment. This result indicates that the nano-Ceria treated cultures had a significantly higher peroxide detoxification ability (Figure 50) and this may also be a significant indicator of its potential protection abilities after ischemic insult.

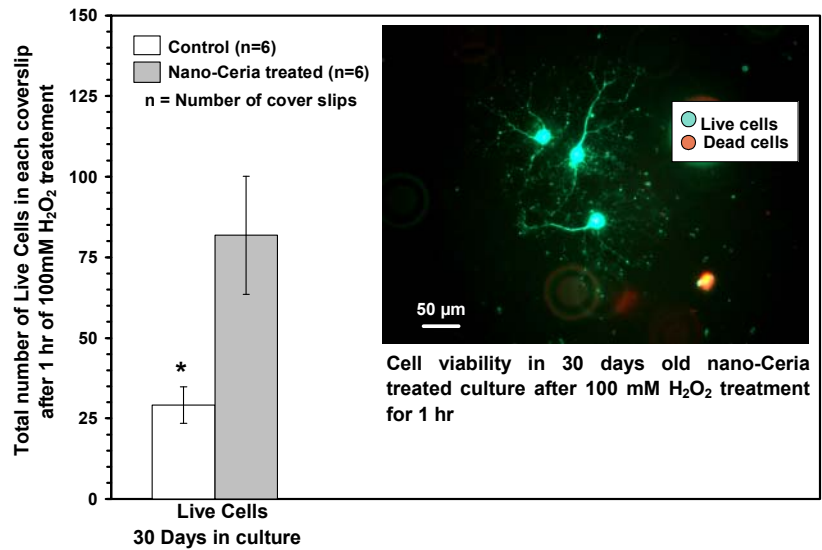


Figure 50. Results After Hydrogen Peroxide-Induced Oxidative Injury in Control and Treated Cultures of Adult Rat Spinal Cord at day 30. Live-dead cell assay after hydrogen peroxide treatment indicates that nano-Ceria treated cultures had a significantly higher number of surviving cells as compared to the control (Data are presented as Mean \pm SE, n = 6; * p < 0.05; ** p < 0.01).

Spinal cord neurons and other CNS neurons are prone to damage due to oxidative stress [28, 29] both in vitro [30] and in vivo [31-33]. To maintain healthy in vitro cultures of spinal neurons and other CNS neurons, several antioxidants are generally used in culture medium. The major source of anti-oxidant molecules in serum-free neuron culture medium is the B27 supplement [23, 34]. B27 contains five antioxidants; vitamin E, vitamin E acetate, superoxide dismutase, catalase, and glutathione [23]. However, the half-life of these antioxidants is limited and they have to be replenished each time the medium is changed to maintain a healthy culture [34]. In nano-Ceria treated cultures, we observed a significant rise in neuron survival as compared to the control culture, which were supplemented only with B27. The auto-regenerative antioxidant properties of a single dose of the autocatalytic nano-Ceria in this in vitro model is the most probable explanation of the significant neuroprotective effect observed in the treated culture.

Based on our cell culture assays, UV-visible spectroscopic studies, hydrogen peroxide-induced oxidative injury assay and our proposed working hypothesis, we conclude that the supplementation of neuronal cultures with a single dose of the Ceria nanoparticles has a significant synergistic effect in a realistic model system of spinal cord injury. Future studies will focus on elucidating the biological mechanism of action of the nano-Ceria. The use of nano-Ceria with other antioxidants may, in the future, prove beneficial for the in vivo mitigation of ischemic events after spinal cord injury as well as possibly being a new therapeutic agent for oxidation injury in the other neurodegenerative diseases or injury.

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CHAPTER 10: TEMPORAL NEUROTRANSMITTER CONDITIONING RESTORES THE
FUNCTIONAL ACTIVITY OF ADULT SPINAL-CORD NEURONS IN LONG-TERM CULTURE.
EXP NEUROL. 2008 JAN;209(1):171-80

Introduction

Spinal cord injury (SCI) and disease are debilitating conditions that have seen limited progress in the full repair of damaged neurons in the CNS [1, 2]. Therefore, much effort has been undertaken to develop *in vivo* models of SCI and study the cellular and molecular mechanisms of synaptic connections and information processing in the spinal cord. However, *in vitro* models of SCI using dissociated adult cells have not been as extensively investigated due to the difficulties associated with culturing adult CNS neurons. Fully functional *in vitro* model systems could be useful not only in spinal cord injury studies but possibly also for models of amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and neuropathic pain. Recent advancements in the culture of adult mammalian spinal cord [3, 4] and brain neurons [5-7] in a completely defined serum-free medium, suggest outstanding potential for answering questions that relate to maturation, aging, neurodegeneration and injury, as well as the ability to screen different novel and putative drug candidates for CNS repair and degenerative diseases of the CNS. Prominent features of the survival of adult CNS neurons in these culture systems have been ascribed to a permissive growth promoting substrate and defined culture medium [3-7].

However, there are few reports on the evaluation of the electrical functionality and regeneration of adult CNS neurons in long-term culture [3, 4, 7, 8]. Recent electrophysiological studies on adult spinal cord neurons in a defined culture indicated that only approximately 30% of the total surviving neurons were electrically active [3, 4]. In all cases, the neurons showed a very weak inward and outward current in voltage clamp studies and only fired single action potentials with limited culture duration [3, 4].

We report here on the development of a robust and long-term culture model of adult rat spinal-cord neurons by the addition of four more growth factors (i.e. VEGF [9, 10], G5 supplement [11], NT-4 [12, 13] and CNTF [14-16]) to a previously developed model [3, 4]. In addition, it was discovered that the electrical functionality of 60% of the neurons could be re-established by long-term temporal incubation of the cultures with serotonin + glutamate (N-acetyl-DL-glutamic acid) followed by acetylcholine-chloride, providing *in vitro* evidence to support the hypothesis that extracellular neurotransmitters may be involved in shaping synaptic circuits *in vivo*.

Methods and Materials

Surface modification of the coverslips:

Glass coverslips (Thomas Scientific 6661F52, 22 × 22 mm² no. 1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 min at 100 mTorr. The DETA (United Chemical Technologies Inc., Bristol, PA, T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904), according to Ravenscroft et al. (1998) [17]. The DETA-coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried (Figure 51).

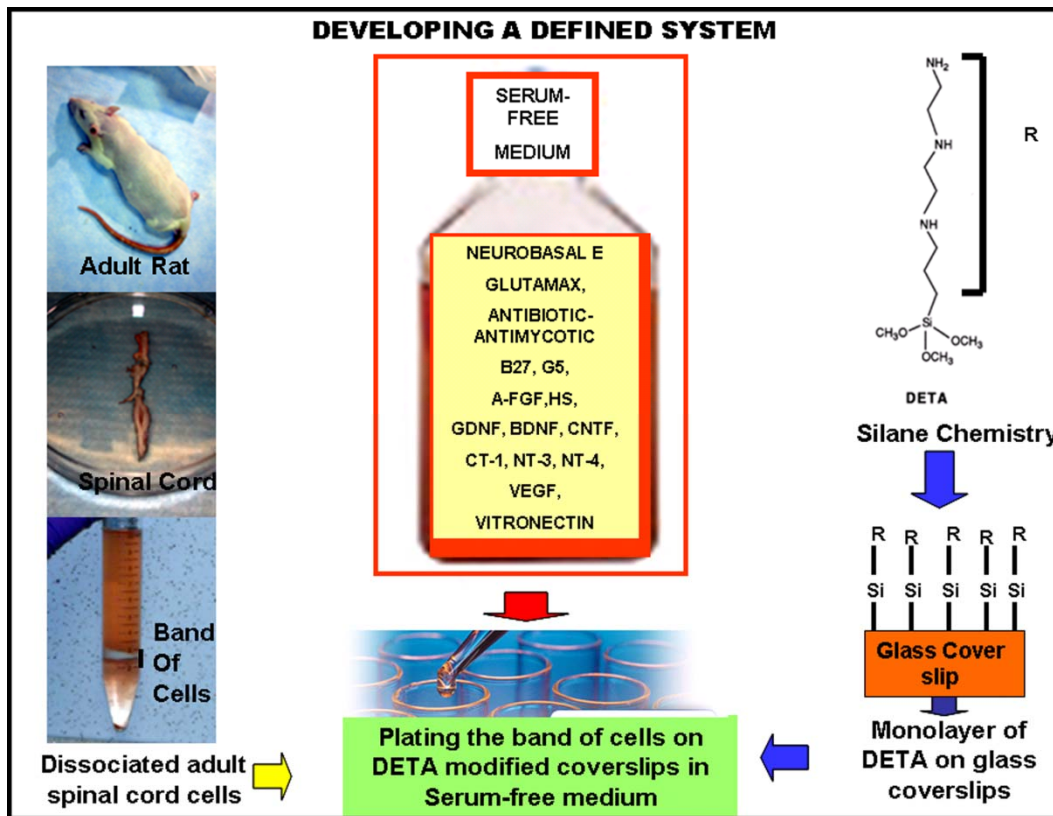


Figure 51. Outline of the defined culture system to study the regeneration of adult mammalian spinal cord neurons.

Surface characterization of the coverslips after coating with DETA

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200) and by X-ray Photoelectron Spectroscopy (XPS) (FISONS ESCALab 220i-XL). The XPS survey scans, as well as high-resolution N 1s and C 1s scans, using monochromatic Al K α excitation, were obtained similar to the previously reported results [3, 4, 17-20].

Isolation and culture of rat spinal cord

Spinal cords were isolated from adult rats (4-6 months old), and the meninges were removed from the spinal cord. The spinal cord was then cut into small pieces and collected in cold Hibernate A (<http://www.brainbitsllc.com>), glutamine (0.5 mM), and B27 (Invitrogen, Carlsbad, CA). Next, the tissue was enzymatically digested for 30 min in papain (2 mg/ml). The tissue was triturated in 6 ml of fresh Hibernate A, glutamine (0.5 mM), and B27. The 6 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505:0.495 [v/v] with Hibernate A–glutamine 0.5 mM–B27) and then made to 15, 20, 25, and 35% (v/v) in Hibernate A–glutamine 0.5 mM–B27 followed by centrifugation for 15 min, using $800 \times g$, at 4° C. The top 7 ml of the supernatant was aspirated. The next 2.75 ml from the major band and below was collected and diluted in 5 ml Hibernate A–B27 and centrifuged at $600 \times g$ for 2 min (Figure 51). The pellet was resuspended in Hibernate A–B27, and after a second centrifugation, the pellet was resuspended in the culture medium (Table 11 shows the specific composition). Complete culture medium change occurred after the first 2–3 d

in culture, and thereafter half of the medium was changed after every 5-6 d [3, 4]. Half of the media was changed on the following days after plating the cells: day 6, 11, 16, 21, 25, 30, 35, 40, 45, 50, 55, 60. Neurotransmitter treatments were started on day 30.

Table 11: Composition of the serum-free medium.

S. No	Component	Amount	Catalogue #	Source
1.	Neurobasal A	500 ml	10888	Gibco/ Invitrogen
2.	Antibiotic-Antimycotic	5 ml	15240-062	Gibco/ Invitrogen
3.	Glutamax	5 ml	35050-061	Gibco/ Invitrogen
4.	B27 Supplement	10 ml	17504-044	Gibco/ Invitrogen
5.	G5 Supplement (100X)	5 ml	17503-012	Gibco/ Invitrogen
6.	VEGF _{165 r Human}	10 µg	P2654	Gibco/ Invitrogen
7.	Acidic FGF	12.5 µg	13241-013	Gibco/ Invitrogen
8.	Heparin Sulphate	50 µg	D9809	Sigma
9.	LIF	10 µg	L5158	Sigma
10.	Vitronectin (Rat Plasma)	50 µg	V0132	Sigma
11.	CNTF	20 µg	CRC 401B	Cell Sciences
12.	NT 3	10 µg	CRN 500B	Cell Sciences
13.	NT 4	10 µg	CRN 501B	Cell Sciences
14.	GDNF	10 µg	CRG 400B	Cell Sciences
15.	BDNF	10 µg	CRB 600B	Cell Sciences
16.	CT-1	10 µg	CRC 700B	Cell Sciences

Throughout the study individual neurotransmitters were added only one time. The details of the neurotransmitter treatments are described in the following paragraphs.

Neurotransmitter application

The three excitatory neurotransmitters glutamate (A8875, Sigma), serotonin (H9523, Sigma) and acetylcholine chloride (A2661, Sigma) were used for the study. N-acetyl-DL-glutamic acid was used as a source for glutamate in the culture because it is naturally occurring in the brain [21]. It is also more stable [22] and we have found that it improved the cell density of the culture.

In the first series of experiments to study the effect of the individual neurotransmitters, 30 days old cultures were treated one time with either 25 μ M N-acetyl-DL-glutamic acid, 25 μ M serotonin or 50 μ M acetylcholine chloride. The electrophysiological evaluations were done after 7 and 14 days of incubation in the neurotransmitters. In the second series of experiments, the combined effect of serotonin (25 μ M) + N-acetyl-DL-glutamic acid (25 μ M) was evaluated. The N-acetyl-DL-glutamic acid + serotonin combination was applied at day 30 and the electrophysiological properties of the cells were evaluated after 7 and 14 days of incubation. In the final part of the experiment, the N-acetyl-DL-glutamic acid (25 μ M) + serotonin (25 μ M) combination was applied at day 30 and followed by the addition of acetylcholine chloride (50 μ M) after 5 additional days. The neuron's electrophysiological properties were evaluated 14 days after the initial treatment of N-

acetyl-DL-glutamic acid +serotonin. During each experiment neurotransmitter solutions were freshly prepared.

Immunocytochemistry

In preparation for staining with anti-neurofilament 150, anti-synaptophysin, anti-nestin protein and the MAP2 a and b antibodies, the coverslips were rinsed free of medium with phosphate-buffered saline (PBS) and fixed for 20 min at room temperature with 10% glacial acetic acid and 90% ethanol. The staining of the coverslips with anti MO-1 and anti-Islet antibody 39.4D5 and ChAT were similarly rinsed free of medium with PBS, but fixed using a different protocol. We added 80 µl of paraformaldehyde (prepared in PBS) in 2 ml of medium for 5 min. This reaction is done by keeping the 6 well plate on top of ice. After 5 minutes, the coverslips were rinsed free of medium with phosphate-buffered saline (PBS) and fixed for 20 min at room temperature with cold fixative (11.1 ml of Formalin+ 89.9 ml of PBS+ 200 µl of Glutaraldehyde+ 4g of Glucose). The rest of the steps for staining remain the same. After 20 minutes of fixing, cells were permeabilized for 5 minutes with permeabilizing solution (50 mM Lysine+ 0.5% Triton X-100+ 100 ml of PBS). After rinsing with PBS, the nonspecific sites were blocked with 5% normal donkey serum and 0.5% Triton X-100 in PBS. The cells were blocked for 2 hours and then the cells are incubated with the primary antibodies for 12 h at 4°C. Cells were incubated overnight at 4° C with either rabbit antineurofilament M polyclonal antibody, 150 kDa (Chemicon, AB1981, diluted 1:1000), anti-nestin (MAB5326, Chemicon, diluted 1:1000), anti-synaptophysin (MAB368, Chemicon, diluted 1:1000),

anti-MAP 2 A and B (MAB364, Chemicon, diluted 1:1000), anti-Choline Acetyltransferase (ChAT, AB143, diluted 1:250), anti-Islet antibody 39.4D5 (Developmental Studies Hybridoma Bank, Iowa City, IA, diluted 1:50), or MO-1 (Developmental Studies Hybridoma Bank, Iowa City, IA, diluted 1:50), in the blocking solution. After incubating overnight, the coverslips were rinsed four times with PBS and then incubated with the appropriate secondary antibodies for 2 h. After rinsing four times in PBS, the cover slips were mounted with Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA) onto slides. The coverslips were observed and photographed using a Ultra VIEW™ LCI confocal imaging system (Perkin Elmer). Controls without primary antibody were negative [3, 4].

Electrophysiology

Whole-cell patch clamp recordings were performed in a recording chamber that was placed on the stage of a Zeiss Axioscope 2 FS Plus upright microscope in Neurobasal culture medium (pH was adjusted to 7.3 with *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulfonic acid [HEPES]) at room temperature. Patch pipettes (6–8 Mohm) were filled with intracellular solution (K-gluconate 140 mM, ethylene glycol-bis[aminoethylether]-tetraacetic acid 1 mM, MgCl₂ 2 mM, Na₂ATP 5 mM, HEPES 10 mM, pH = 7.2). Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon, Union City, CA) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis was performed with pClamp 8 (Axon) software. Sodium and potassium currents were measured in voltage clamp mode

using voltage steps of 10 mV from a -70 mV holding potential. Whole-cell capacitance and series resistance was compensated and a p/6 protocol was used. The access resistance was less than 22 Mohm. Action potentials were measured with 1 s depolarizing current injections from the -70 mV holding potential [3, 4, 18-20].

Statistics

Chi-squared test: Any significant differences ($p = 0.05$) between treatments as compared to the control were quantified using the double classification Chi-squared test (Table 10.2).

ANOVA: For pairwise comparisons of the means, we used a one-way ANOVA and statistical tests assuming unequal variance (Tamhane's test) (Table 10.3).

Results and Discussion

Adult spinal-cord neuron regeneration experiments were carried out in a defined culture system which consisted of an empirically derived serum-free medium, synthetic cell growth promoting silane substrate and a well defined culture technique (Figure 51). The detailed composition of the serum-free medium is presented in (Table 11). The culture system was initiated by plating the dissociated adult rat spinal-cord cells on a synthetic, patternable [17], cell growth promoting organosilane substrate, N-1[3-(trimethoxysilyl)propyl]-diethylenetriamine (DETA) [3, 4, 18-20, 23]. Detailed protocols for the surface chemical modification of the substrate, characterization of the substrate, rat dissection, cell isolation and cell culture are discussed elsewhere [3, 4] and described in detail in the methods section.

The advantages of a synthetic silane substrate are its reproducibility and suitability for high resolution patterning to allow the development of engineered networks [17],[3, 4, 18, 19, 23] as well as the ability to couple specific extracellular matrix molecules and different contact signaling molecules to systematically study the specific role of such molecules in remyelination, neurodegeneration, and axonal growth inhibition during spinal cord injury. The ability to create functional in vitro networks of different types of neurons as well as neurons and muscle will allow detailed study of how these networks can be created or regenerated without having to observe the processes in vivo. Now the potential to recreate circuits in a defined system with adult cells extends

this capability enormously. These results reported here, now allow the recreation of this active network in vitro.

In this culture model we have added four additional growth factors (i.e. VEGF, G5 supplement, NT-4 and CNTF) to a previously developed model [3, 4]. The present culture model, with four additional growth factors, promoted long-term survival (8-10 weeks) of the adult spinal cord cells (Table 10.1). Previously these individual factors (i.e. VEGF [9, 10], G5 supplement [11], NT-4 [12, 13] and CNTF [14-16]) had been shown to improve the survival of spinal cord neurons and glial cells either or both in vivo and in vitro. Possibly, these growth factors play a synergistic role in promoting the long-term survival of the regenerating adult spinal cord neurons in culture.

The neurons began their regeneration process within 24 h of plating the cells (Figure 52, upper panel) and this was characterized by the co-expression of nestin and neurofilament-150 proteins by the majority of the neurons between day 1-3. By day 4, the neurons only expressed neurofilament-150 and other neuron specific markers, with nestin expression lost by day 4 (Figure 52 lower panel). Co-expression of nestin and neurofilament-150 during the early stages of cell growth suggests that these cells may undergo an embryonic 'reprogramming' to allow for the regeneration of axonal and dendritic processes.

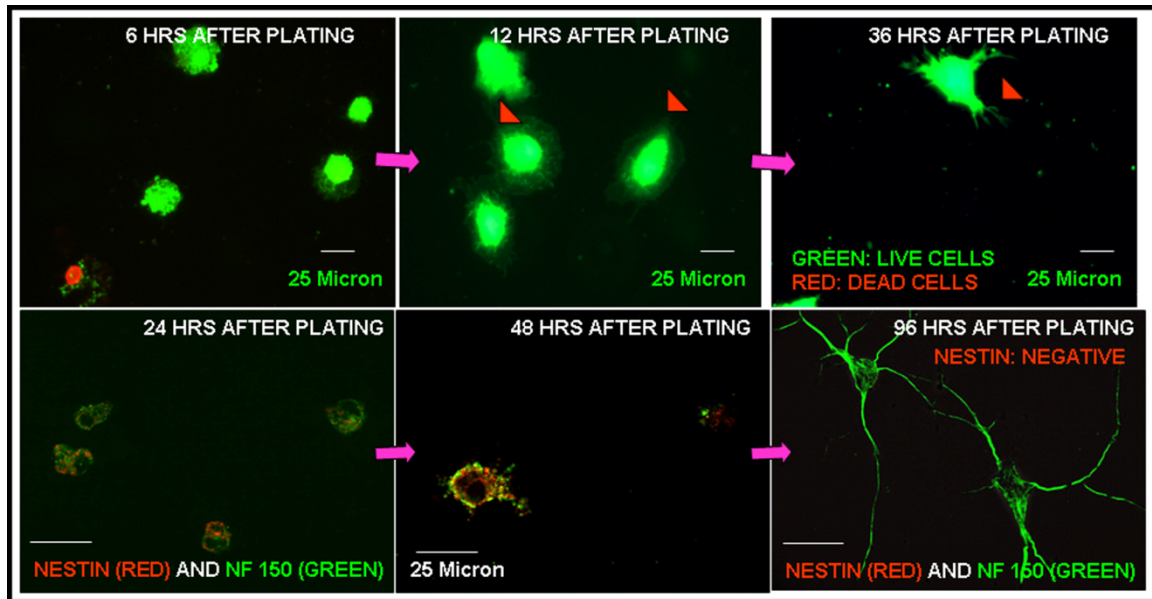


Figure 52. Immunocytochemical evidence of the early events during the initiation of the regeneration process utilizing nestin and neurofilament-150. Upper panel. The regeneration process was initiated during the first 24 hours of cell plating and the live/dead assay indicates the majority of the plated cells are alive. Lower panel. Early regeneration events are characterized by co-expression of the nestin and neurofilament 150 proteins by most neurons between day 1-3. By day 4, the neurons only express neurofilament-150 and other neuron specific markers, as the nestin expression was lost by day 4.

Specific areas of the spinal cord were not selected for isolation and culture, suggesting that the culture contains a mixture of ventral horn motoneurons, dorsal horn neurons and interneurons. In addition, the culture contained 30-40% of GFAP positive cells although the proliferative potential of these cells was limited by the composition of the defined culture conditions used. The cultures were immunocytochemically characterized for the different cell types present at two different time intervals, day 35 and day 45 in culture. We used 6 different neuron specific antibodies (Islet-1, ChAT, MO-1, NF-150, MAP-2 and synpatophysin) for the immunocytochemistry. The results are presented in Figure 53, upper and lower panels. At day 35, most of the neurons exhibiting a smooth-appearing large soma, a multi-polar dendritic tree and a long axon, were later found to be electrically active. These neurons stained positive for Islet-1, MO-1 and ChAT (Figure 53 A, B, C, D), the three ventral horn spinal-cord motoneuron markers. In addition, the neurons expressed MAP-2 a and b, NF-150 proteins and synaptophysin (Figure 53 E, F, G, H).

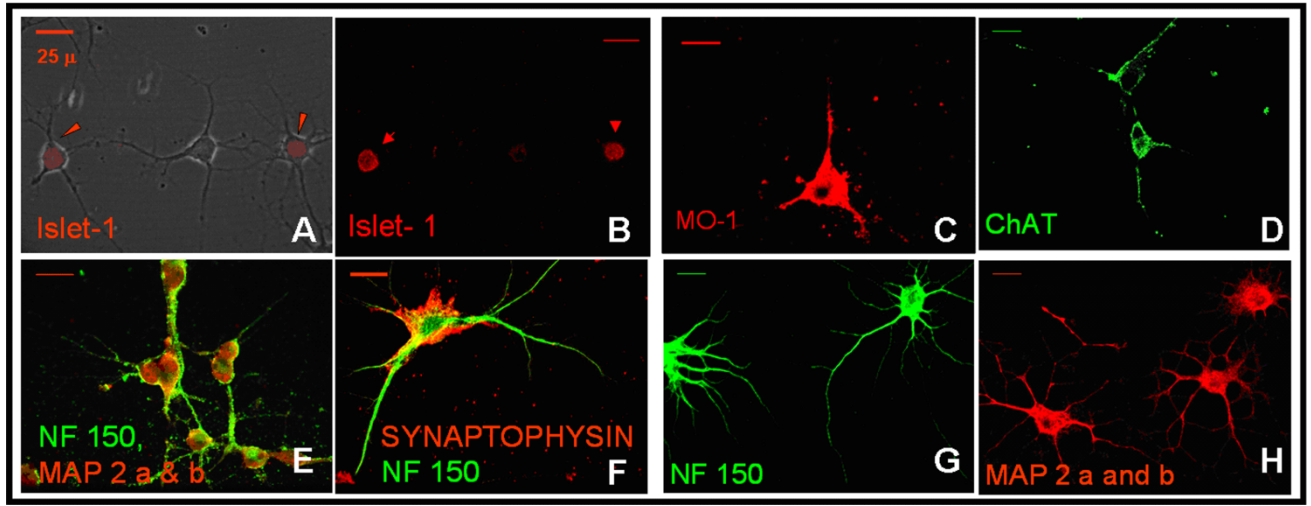


Figure 53. Immunostained cultures at day 35 utilizing different neuron specific antibodies. A. Phase coupled with fluorescence micrograph showing neurons stained with ISLET-1 antibody (a putative motoneuron marker). B. Fluorescent staining of the ISLET-1 positive cells shown in figure A. C. Neurons stained with MO-1 antibody (a putative motoneuron marker). D. Neurons stained with ChAT antibody (a putative motoneuron marker). E. Neurons double-stained with MAP 2a and b and NF 150 antibodies. F. Neurons double-stained with synaptophysin and NF 150 antibodies. G. Neurons stained with NF 150 antibody. H. Neurons stained with MAP 2, a and b antibody.

Whole cell patch clamp experiments were used to evaluate the electrical activity of the neurons. The duration of the regeneration process was approximately 25-30 days and led to a reticular network formation by day 35-40. Hence, we specifically choose two time points past this time period to study the electrical properties, day 37 and 44. In order to minimize electrophysiological heterogeneity, cells which were morphologically identical to cells previously characterized by immunocytochemistry were studied. Figure 54 shows representative pictures of the neurons selected for the study. Morphologically, the neurons selected resembled ventral horn motoneurons. For comparison, the properties of several large cells with radial symmetry, which most resembled astrocytes, oligodendroglia or ameboid microglia, were studied.

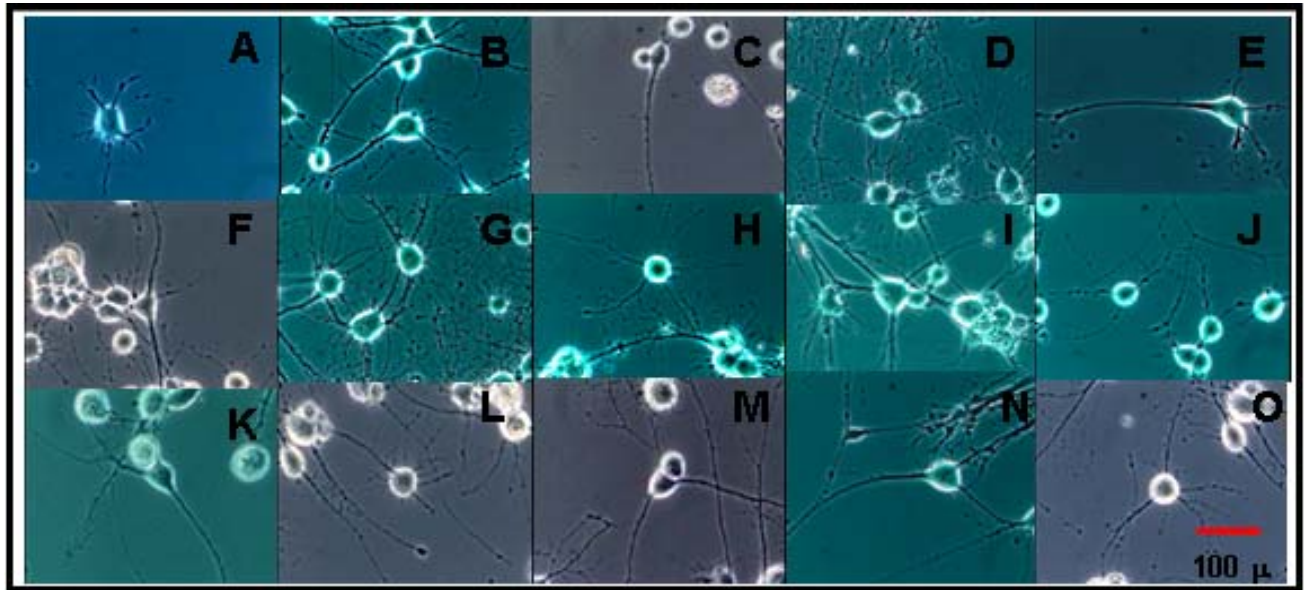


Figure 54. Representative phase-contrast pictures of the cells which were used to quantify the electrical properties. A and B. Phase pictures of the neurons in control culture at day 44. C. Phase pictures of the neurons after glutamate treatment at day 37 (G37). D. Phase pictures of the neurons after glutamate treatment at day 44 (G44). E. Phase pictures of the neurons after serotonin treatment at day 37 (S37). F. Phase pictures of the neurons after serotonin treatment at day 44 (S44). G. Phase pictures of the neurons after acetylcholine chloride treatment at day 37 (A37). H. Phase pictures of the neurons after acetylcholine chloride treatment at day 44 (A44). I. Phase pictures of the neurons after glutamate+serotonin treatment at day 37 (GS37). J and K. Phase pictures of the neurons after glutamate+serotonin treatment at day 44 (GS44). L, M, N and O. Phase pictures of the neurons after glutamate+serotonin followed by acetylcholine chloride treatment at day 44 (GSA44).

Initially, the electrical properties of the neurons in long-term culture were evaluated as controls. Similar to what has been reported previously, at day 37 (C37) and 44 (C44), 29% and 28% of the cells exhibited single action potentials (AP), respectively (Table 12).

Table 12: Comparison of the total number of cells patched and the number of cells which exhibited APs in control (C37, C44), glutamate treated (G37, G44), serotonin treated (S37, S44), acetylcholine chloride treated (A37, A44), glutamate+serotonin treated (GS37, GS44), and glutamate+serotonin→ acetylcholine chloride treated (GSA44). 37 and 44 indicates 7 and 14 days after culturing the cells in the presence of neurotransmitters respectively. Percentages are indicated in parentheses.

	Total number of cells patched	Number of cells which did not fire any action potential (NP)	Number of cells which fired single action potentials (SAP)	Number of cells which fired double action potentials (DAP)	Number of cells which fired multiple action potentials (MAP)
C37	24	17	7 (29.1%)	-	-
C44	49	35	14 (28.5%)	-	-
G37	42	28	14 (33.3%)	-	-
G44	14	8	6 (42.2%)	-	-
S37	17	10	7 (41.1%)	-	-
S44	25	14	11 (44.0%)	-	-
A37	30	25	5 (16.6%)	-	-
A44	31	25	6 (19.3%)	-	-
GS37	20	10	10 (50.0%)	-	-
GS44	27	11	14 (56.0%)	2 (12.5%)**	-
GSA44*	107	42	57 (60.7%)	5 (7.6%)**	3 (4.6%)***

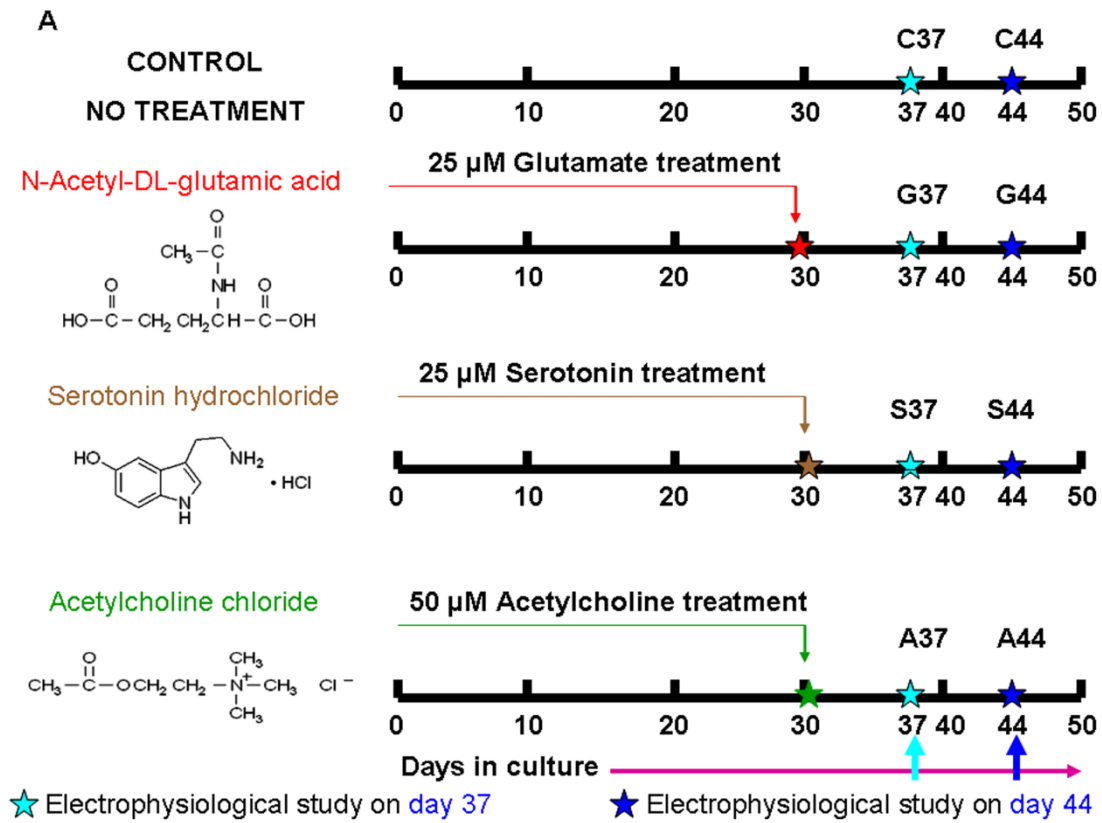
*A double classification Chi-squared test was used to test for multiple categories of data. We compare the X^2 value with a tabulated χ^2 with one degree of freedom. Our calculated X^2 exceeds the tabulated χ^2 value (3.84) for $p = 0.05$. We conclude that multiple neurotransmitter applications show significantly more electrically active cells as compared to the control.

**Percentage of neurons as compared to the total number of electrically active neurons which fired double APs.

*** Percentage of neurons as compared to the total number of electrically active neurons which fired multiple APs.

Research during last two decades have indicated that during the neural circuit development in the spinal cord, retina, and hippocampus, the electrical stimulation originates due to spontaneous network activity and paracrine neurotransmitter signaling involved in sculpturing the network activity [24-26]. Based on this, we speculated that administration of neurotransmitters could mimic the similar developmental condition and could further improve the functional characteristics of the regenerating adult spinal cord cells.

We selected three excitatory neurotransmitters, serotonin, glutamate and acetylcholine-chloride for study. The rational for selecting these three neurotransmitters was to test the hypothesis of whether the application of extracellular neurotransmitters improve the electrical properties of the regenerating adult spinal cord neurons. The detailed protocol, dosages and timing of the neurotransmitter application has been described in the methods section and is outlined in Figure 55 A, B.



B

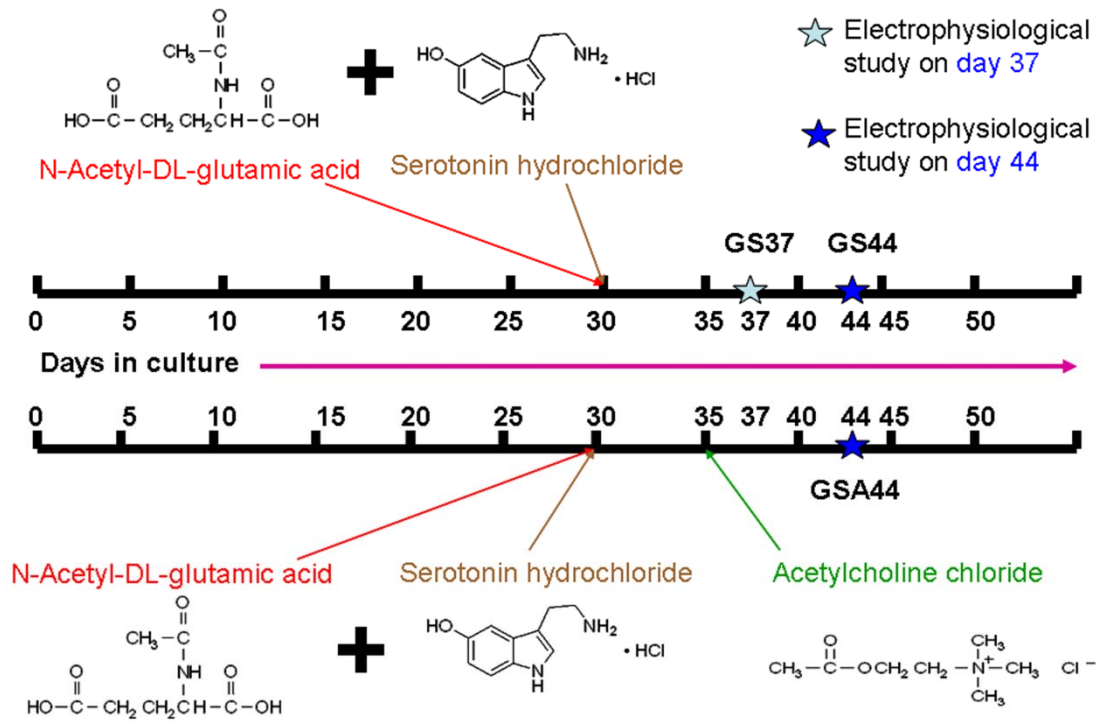


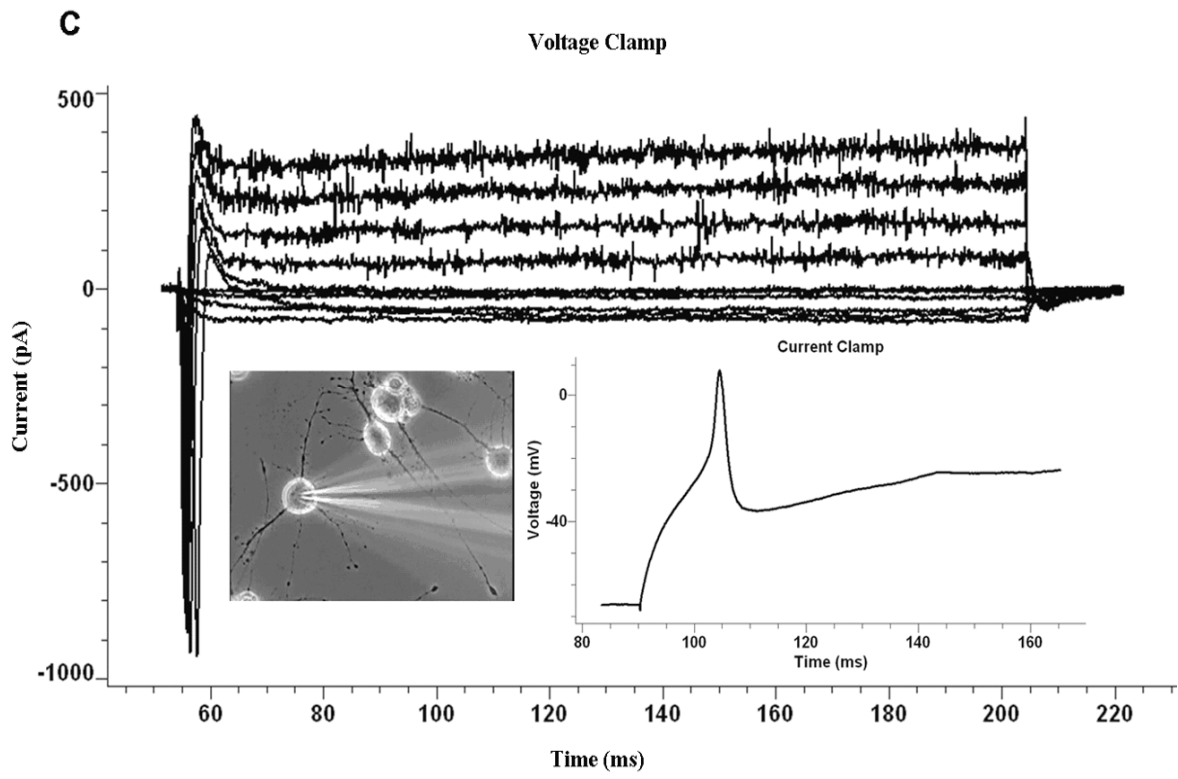
Figure 55. Electrophysiological recordings from glutamate+serotonin→acetylcholine chloride (GSA44) treated cultures. A. Scheme for single neurotransmitters application. B. Scheme for multiple neurotransmitters application.

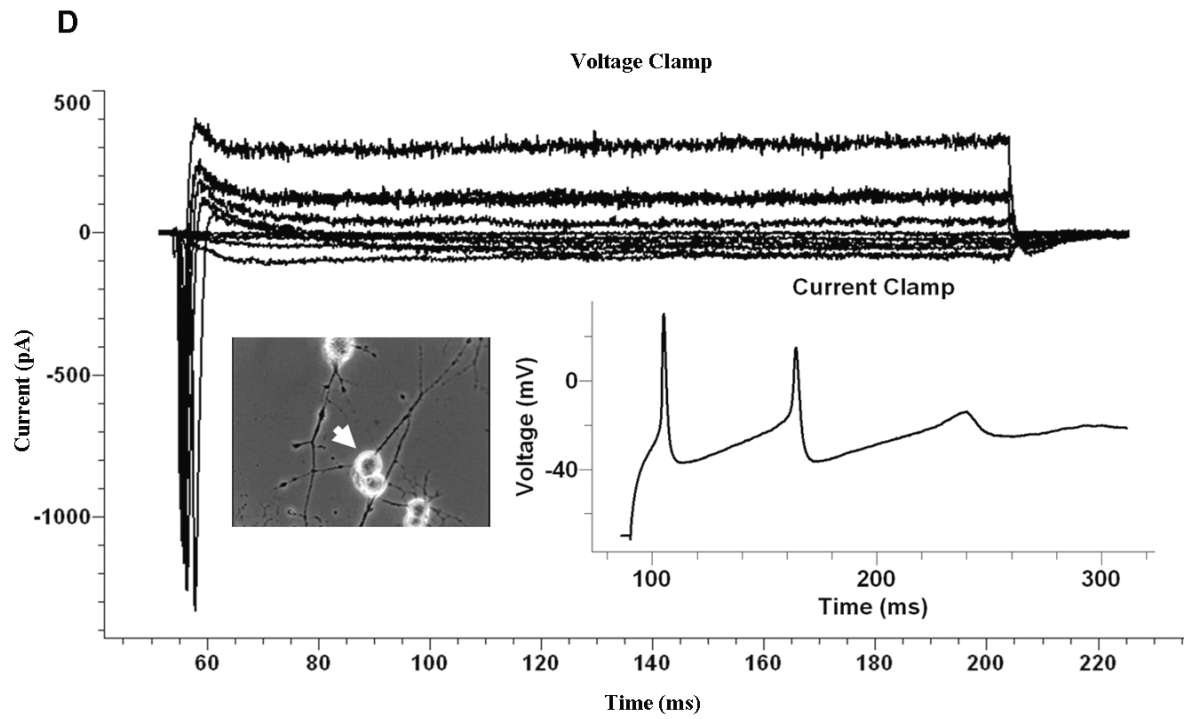
The first set of electrophysiological experiments was completed by incubating the cultures separately in three different neurotransmitters: glutamate, serotonin, and acetylcholine-chloride (Table 12).

The neurotransmitter treatments were performed on 30 day old cultures. The electrical properties were evaluated at two different time intervals, 7 and 14 days after neurotransmitter incubation. 33% of the cells fired single AP's following 7 days of glutamate incubation (G37). There was a 10% increase in the number of cells firing AP's following 14 days of glutamate incubation (G44). 41% and 44% of the total cells exhibited single AP's after 7 (S37) and 14 (S44) days of serotonin treatment, respectively. Compared to the control, there was a decrease in the number of cells exhibiting AP's after 7 (16% for A37) and 14 (19% for A44) days of acetylcholine chloride incubation.

In the second set of experiments, the cultures were incubated with multiple neurotransmitters. 50% of the cells fired single AP's when co-administered glutamate+serotonin (GS37) and then incubated in culture for 7 days. Whereas, after 14 days of incubation (GS44), 56% of the cells fired a single AP, and 2 out of 16 neurons fired double AP's. To determine if the addition of acetylcholine-chloride after co-administration of glutamate+serotonin could influence the electrical properties of the recovering neurons, acetylcholine-chloride was added after 5 days. The electrical properties were then evaluated at day 14 after the initial co-administration of glutamate+serotonin.

We observed a significant improvement ($p = 0.05$) in the electrical properties of the regenerating neurons using this treatment regime. 60% of the neurons that followed this temporal application of the three neurotransmitter treatments (GSA44) fired either single, double or multiple action-potentials (Figure 56 A, B, C). The results are summarized in Table 12 and a double classification Chi-squared test was used to quantify any significant difference ($p = 0.05$) between different treatments as compared to the control.





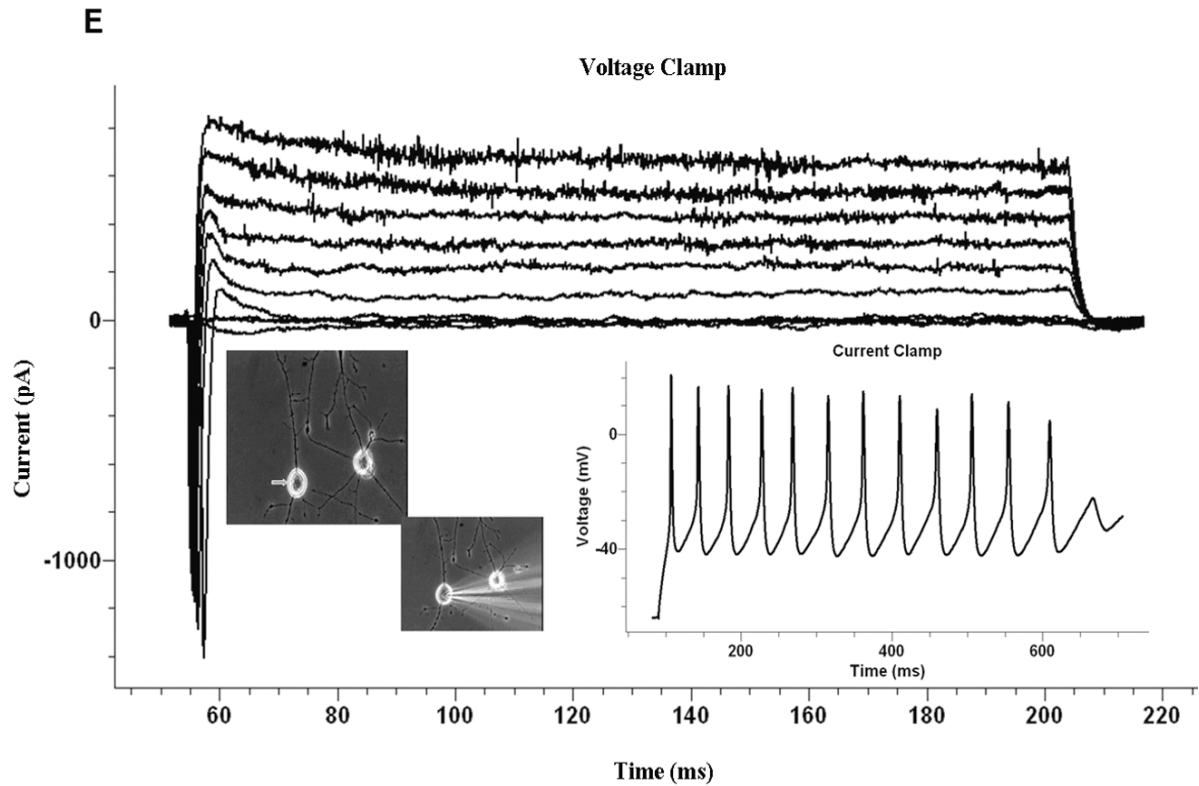


Figure 56. A. Representative trace for voltage and current clamp of a neuron firing a single action potential after multiple neurotransmitter applications at day 44. B. Representative trace for voltage and current clamp recordings of a double action potential firing neuron after multiple neurotransmitter applications at day 44. C. Representative trace for voltage and current clamp recordings of a neuron firing multiple action potentials after multiple neurotransmitter applications at day 44.

Interestingly, the inward current was the only parameter that differed significantly ($p = 0.01$) between the different groups of neurons (Table 13). As compared to the neurons in the control culture (C37), the serotonin (S44), glutamate+serotonin (GS44), and glutamate+serotonin→acetylcholine chloride (GSA44) treated neurons expressed significantly more inward current. We also observed more inward current from the neurons which were incubated in glutamate+serotonin→acetylcholine chloride for 14 days (GSA44) as compared to those cultures which were incubated in either serotonin (S37), serotonin (S44), acetylcholine chloride (A37) or acetylcholine chloride (A44).

Similarly, an increase in inward current was also exhibited by neurons which were incubated in glutamate+serotonin for 14 days (GS44) as compared to those cultures which were incubated in either S37, S44, G44, A37, or A44. There was also a significant improvement in the electrical properties of the neurons which were incubated for 14 days in glutamate+serotonin (GS44) as compared to those which were only incubated for 7 days (GS37) (Table 13). These results illustrate that full functional recovery was achieved after 14 days following treatment of the cultures with the multiple neurotransmitter protocol.

The cultures were also analyzed for differences in resting potential, membrane resistance, membrane capacitance, inward current, outward current, AP height and AP width. These results are summarized in Table 13 and expressed as mean \pm SE. One-way ANOVA and statistical tests,

assuming unequal variance (Tamhane's test), were used for pairwise comparisons of the mean. There were no significant differences ($p = 0.01$) in resting potential, membrane resistance, membrane capacitance, outward current, AP height and AP width between the different experimental groups of the cultured neurons.

Table 13: Comparison of the electrical properties of the neurons which exhibited APs in control (C37, C44), glutamate treated (G37, G44), serotonin treated (S37, S44), acetylcholine chloride treated (A37, A44), glutamate+serotonin treated (GS37, GS44), and glutamate+serotonin→acetylcholine chloride treated (GSA44). Since the neurotransmitters were added on day 30 after the cells were plated, the numbers 37 and 44 indicate 7 and 14 days respectively after culturing the cells in the presence of neurotransmitters. The values are expressed as Mean \pm SE.

	C37	C44	G37	G44	S37	S44	A37	A44	GS37	GS44	GSA44	P
Days in vitro	37	44	37	44	37	44	37	44	37	44	44	
Number of neurons (n)	7	14	14	6	7	11	5	6	10	16	65	
Resting potential (Mv)	51.7 ±3.1	48.2 ±2.9	48.9 ±3.2	50.4 ±4.4	50.3 ±1.5	48.9 ±1.3	52.4 ±2.1	52.5 ±1.7	52.8 ±2.8	46.2 ±3.3	44.2 ±1.3	NS
Input resistance (mΩ)	163.3 ±12.1	169.1 ±11.1	124.1 ±16.6	110.3 ±16.5	130.9 ±9.2	136.9 ±12.9	128.2 ±7.1	131.7 ±10.7	144.5 ±8.9	278.9 ±74.0	237.9 ±23.1	NS
Capacitance (pF)	18.3 ±1.9	17.0 ±1.9	15.8 ±1.1	14.8 ±1.2	15.71 ±1.5	15.4 ±1.5	15.4 ±1.6	18.5 ±1.9	17.2 ±2.8	18.9 ±1.4	17.7 ±0.9	NS
Peak inward current (pA)	657.1 ±39.9	681.4 ±134.6	674.8 ±127.9	737.2 ±69.4	402.1 ±43.7	359.1 ±33.6	450.0 ±44.7	525.0 ±70.4	713.9 ±95.2	1376.4 ±105.2	1090.4 ±79.9	= 0.01
Peak outward current (pA)	770.7 ±43.1	833.5 ±110.3	883.6 ±166.2	855.0 ±77.3	829.3 ±70.2	745.5 ±67.5	605.0 ±37.4	625.0 ±61.6	768.6 ±83.2	1219.8 ±117.7	930.1 ±41.9	NS
Action potential height (mV)	26.4 ±2.4	16.6 ±1.3	18.1 ±2.3	20.2 ±3.4	20.7 ±1.5	16.9 ±1.3	18.8 ±2.6	20.2 ±2.4	18.4 ±1.8	25.6 ±2.4	23.84 ±1.1	NS
Action potential width (ms)	3.9 ±0.2	4.4 ±0.2	5.3 ±0.7	3.7 ±0.2	4.2 ±0.3	4.6 ±0.3	4.7 ±0.4	4.7 ±0.4	5.4 ±0.5	5.3 ±0.3	5.80 ±0.2	NS

The three neurotransmitters which were used for this study are all excitatory neurotransmitters and depolarize the cell membrane. Synaptically released glutamate, serotonin, acetylcholine and other neurotransmitters have been studied in detail [27]. However, very little in vivo information has been available about the origin and function of the pool of glutamate [28, 29], serotonin [30], acetylcholine [31-33] and other neurotransmitters which appear to be present in micromolar concentrations in the extracellular space outside the synaptic cleft [34]. Our results support one hypothesis in that extracellular neurotransmitters may be involved in shaping synaptic activity in vivo [28, 29, 35]. Thus, these results raise the distinct possibility that this strategy could also be exploited to differentiate stem cells to form neurons as well as be applied to establish functional recovery of damaged neurons in vivo.

Our results now provide one of the missing components that enable full adult CNS neuron regeneration and demonstrate that the adult CNS neurons can re-establish their electrophysiological functionality in a fashion similar to that found during embryonic development. In addition, we have demonstrated for the first time that it is necessary to have an application of neurotransmitters, as well as growth factors, to enable the injured adult CNS neurons to regain their full electrical functionality. This new ability to culture functional adult CNS neurons will be of importance in unlocking molecular pathologies to neurological disorders such as SCI, ALS, Parkinsons and Alzhiemers which are specific to adult differentiated neurons.

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CHAPTER 11: GENERAL DISCUSSION

Translational research is currently a priority of the National Institutes of Health and other national and state funding agencies and foundations. In the context of tissue engineering and regenerative medicine, translational research can refer to the transformation of *in vitro* tissue engineering and regenerative medicine technologies and discoveries into practical solutions. One of the key challenges in translating the *in vitro* tissue engineering and regenerative medicine results for clinical applications is the lack of defined model test beds. The non-availability of defined model test-beds leads to variation in results from laboratory to laboratory. The variation arises due to the following discrepancies:

1. Lack of uniform cell isolation and cell culture methodology.
2. Poor understanding of the growth factor requirements of the cells and minimum use of micro-fluidics platform for growth factor delivery.
3. Non-availability of defined mediums and heavily relying on serum supported cultures.
4. Lack of research in understanding cell-cell interactions.
5. Minimum use of surface engineering tools to quantify cell-matrix interactions so as to develop advance biomaterials.
6. The technological barrier for using photolithography and other surface engineering tools for controlling cell shape and orientation so as to create two and three-dimensional patterned substrates.
7. Two or three cell type interaction studies are lacking due to the complexity in handling multiple

cell types in one system. Micro-fabrication technologies in cell culture laboratories have not been widely used to create compartmentalized culture systems with micro-fluidics channels for nutrient and growth factor transfers.

One approach which has been taken to remove these discrepancies is to integrate the knowledge of cell biology techniques and growth factor biology with modern surface engineering technology, micro-fabrication technology, micro-fluidics platforms and inter-face technology. The surface engineering tools provide the state of art technologies to characterize substrates, engineer substrates at nanometer dimensions and manipulate the surface properties of the substrates. Micro-fabrication technology offers the scope to develop small bio-hybrid tools and micro-compartment chambers to study the interactions of different cell types so as to predict the function of interacting organ systems of our body. Micro-fluidics platforms offers the scope to control the delivery of different factors involved in cell growth. Finally interface technology assists in integrating these different technologies to create functional bio-hybrid devices. In future, such bio-hybrid devices will be extremely helpful not only for translational research but also addressing basic problems in biology and medicine and for developing prosthetic devices. This dissertation research work is an effort in that direction where an attempt has been made to integrate cell culture technology, growth factor biology, surface engineering strategies, micro-fabrication technology and interface technology to develop an engineered test bed to study the real time interaction of motoneuron-skeletal muscle construct.

In this body of work, the different tissue engineering strategies and technologies have been addressed to enable the recreation of a *in vitro* cellular prototype of the stretch reflex circuit with special emphasis on building the motoneuron to muscle segment of the circuit. In order to recreate the motoneuron to muscle segment of the stretch reflex arc, a successful methodology to tissue engineer skeletal muscle and motoneuron was essential. Hence the recreation of the motoneuron to muscle segment of the stretch reflex circuit was achieved in two parts. In the part 1 (Chapters 2-5), the challenges in skeletal muscle tissue engineering were examined [1, 2]. In part 2 (Chapters 6-7), apart from tissue engineering the motoneuron[3] to muscle segment, the real time synaptic activity between motoneuron and muscle segment were studied using extensive video recordings[4]. In part 3 (Chapters 8-10), an innovative attempt had been made to tissue engineer the adult mammalian spinal cord neurons so that in future this technology could utilized to replace the embryonic neurons used in the stretch reflex circuit with adult neurons[5-7]. The advantage of using adult neurons is that it provides a powerful tool to study older neurons since these neurons are more prone to age related changes, neurodegenerative disorders and injuries. In the following paragraphs, the consequences of the individual parts of this work had been discussed. Furthermore, some challenging questions for future research had been enumerated.

Skeletal muscle differentiation is a common model system to study differentiation process in cell biology[8]. Although it is a very well studied model, limited success had been achieved in generating different muscle phenotypes in culture[9-11]. This limited success was mostly due to

non-availability of a defined culture model. All the culture models relied heavily on the use of serum[12-15]. This further posed a serious challenge in successful tissue engineering of the skeletal muscle for therapeutic applications. In order to create tissue engineered constructs for therapeutic applications, it is essential to have a defined medium formulation. So there existed a gap in knowledge between basic cell biology and tissue engineering. In order to bridge this gap in knowledge, there was a serious need to revisit the skeletal muscle differentiation problems from a tissue engineering perspective. Part 1 aimed at exploring the skeletal muscle tissue engineering problem from a tissue engineer's approach. Hence four key goals were set. The goals were as follows:

1. Developing a uniform skeletal muscle cell isolation and cell culture methodology.
2. Understanding the growth factor requirements of the skeletal muscle cells and developing defined mediums.
3. Understanding the biocompatibility of the muscle cells on a synthetic substrate.
4. Integrating muscle cells with a cantilever based bio-hybrid device.

The work was documented in four independent experiments (Chapters 2-5). Through this work, a robust serum-free medium was developed which promoted skeletal muscle differentiation[1, 2]. Myotubes exhibited expression of both embryonic and neonatal myosin heavy chain proteins. Formation of the excitation-contraction (E-C) coupling apparatus was documented. Myotubes exhibited physiological electrical properties[2]. Myotubes were also successfully integrated into

cantilever based bio-hybrid device[2, 16]. This work has thrown some challenging questions for future studies. Some of those questions had been enumerated below:

1. How to manipulate growth factor requirements so as to generate different fast and slow twitch muscle phenotypes?
2. How to develop intrafusal fibers for muscle spindle development?[17]
3. How to pattern myotubes in two and three dimensional matrices for tissue engineered constructs?[18]
4. Could we use a similar cell culture technology to differentiate the satellite cells to form adult muscle phenotypes?

Currently efforts are going on in order to address some of these challenging questions. This work had bridged the gap in knowledge that has existed between basic skeletal muscle cell biology and skeletal muscle tissue engineering. In the future this novel medium formulation could be used for tissue engineering of skeletal muscle.

In part 2, the key goal was to integrate the tissue engineered muscle developed in part 1 with embryonic motoneurons, so as to recreate the motoneuron to muscle segment of the stretch reflex arc. In order to achieve this goal, there was a need to co-culture the skeletal muscle cells with motoneurons. The major challenges in co-culturing nerve and skeletal muscle were the complexity of the culture techniques and extensive use of serum in the co-culture. Previously all known models followed a complex culture technique and used serum in the medium[19]. Hence there was

no known defined medium which could be successfully used to coculture mammalian nerve and muscle cells. This necessitated a need to develop a defined system so as to successfully tissue engineer the motoneuron to muscle segment of the stretch reflex arc so as to study the neuromuscular junction. This work was documented in two independent experiments (Chapters 6-7). A significant biotechnological advancement was made in chapter 6, by using a synthetic substrate and creating a minimalist serum-free defined system to tissue-engineer the mammalian neuromuscular junction. A co-culture model of rat mammalian nerve and muscle cells was achieved which demonstrated the formation of functional neuromuscular junctions[4]. Extension of this work in experiment B, further demonstrated that expression of more mature myosin heavy chain proteins in muscle is probably regulated by nerve innervations. This system has given birth to series of challenging questions for future investigations:

1. How this model could be integrated into a micro-cantilever based bio-hybrid device?[1, 2, 16]
The goal for such integration is to study the encoded electrical signals generated by the motoneuron, which controls the different muscle movements. Furthermore, using these signals to design the next generation of robots.
2. How this information could be translated in finding therapies for spinal cord repair, amyotrophic lateral sclerosis (ALS) and other nerve-muscle disorders and most importantly in regenerative medicine?

Part 3, addressed one of the most intriguing questions: Could we make bio-hybrid circuits of stretch reflex arc and other complex central nervous system circuits using adult mammalian spinal cord neurons? This question led to another challenging problem of modern neuronal medicine that is “How we could protect adult mammalian spinal cord nerve cells, either following injury or in neurodegenerative diseases”. One of the key prerequisite in *in vitro* biology to address this question is to create a serum-free defined *in vitro* cell culture model of adult mammalian spinal cord cells. Such a model will not only help in understanding the challenges in incorporating adult spinal cord neuron in the stretch reflex circuit but would be helpful in understanding the growth factor requirements to make these adult neurons survive as well as to screen different novel drugs for spinal cord repair and neurodegenerative disorders of spinal cord like amyotrophic lateral sclerosis (ALS). In this work, an attempt had been made to develop a culture model to grow adult rat spinal cord neurons using different growth factors, hormones, anti-oxidant nanoparticles and neurotransmitters. This work was documented in three independent experiments (chapters 8-10). In chapter 8, for the first time, it was demonstrated that it was feasible to culture adult mammalian spinal cord neurons in a chemically defined, serum-free medium and on a synthetic substrate[6]. 30% of the total neurons were found to be electrically active and the culture survived for 3-4 weeks. This offered a simple *in vitro* tool to study both aging spinal cord neurons and spinal cord injury *in vitro*. Further this finding raised some challenging questions for future research:

1. How to enhance the survival of the neurons and how to regain the electrical activity of most of the neurons ?

2. Could these adult spinal cord neurons be patterned in specific geometries to study the network behavior of adult spinal cord neurons?
3. Could these adult neurons be incorporated in functional hybrid devices to create next generation of neuro-prosthetic devices?
4. Could these adult neurons be remyelinated in the culture with oligodendrocytes and Schwann cells?
5. Could human spinal cord cells be regenerated using a similar cell culture technology?

These questions set the goal for the next experiment. This goal was to enhance the survival of the neurons in the culture. In chapter 9, the survival of the adult spinal cord neurons was enhanced by using a novel, nanoparticle antioxidant; Cerium oxide. The auto-catalytic, anti-oxidant behavior and biocompatibility of cerium oxide nanoparticles were evaluated in increasing the survival of adult, mammalian spinal cord neurons in culture[7]. This *in vitro* experimental evidence raised the possibility that antioxidant cerium oxide nanoparticle could be administered in animals to evaluate its efficacy as a nanomedicine for spinal cord repair. Although, significant improvement was made in the neuronal survival only 30% of the neurons were electrically active. Hence there was a need to undertake further investigations to improve the electrical activity of the neurons in culture. So there was a need to discover the missing components in the culture system, which could enhance the electrical activity of the adult spinal cord neurons. Chapter 10 investigated methods to regain the electrical maturity of most of the neurons in the culture. In chapter 10, for the first time, it was

demonstrated that it is necessary to have an application of multiple neurotransmitters in a temporal fashion, in addition to just growth factors, to enable the injured adult CNS neurons to regain their full electrical functionality[5]. Due to multiple neurotransmitter treatment, 70% of the neurons were active in the culture system. These results support one hypothesis in that extracellular neurotransmitters may be involved in shaping synaptic activity *in vivo*. Thus, these results raise the distinct possibility that this strategy could also be exploited to differentiate stem cells to form neurons as well as be applied to establish functional recovery of damaged neurons *in vivo*. In future studies, a mechanistic understanding is essential to understand the exact role by which neurotransmitters help in functional recovery of adult spinal cord neurons in culture.

This study has successfully demonstrated the recreation of the motoneuron to muscle segment of the stretch reflex arc and further demonstrated the successful tissue engineering strategies to grow adult mammalian spinal cord neurons. The different cell culture technologies developed in these studies could be used as powerful tools in nerve-muscle tissue engineering, neuro-prosthetic devices and in regenerative medicine.

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