# NEURONAL CELL CULTURE MATRIX FOR BETTER MAINTENANCE AND SURVIVAL OF NEURONAL CELL CULTURES IN TISSUE CULTURE.

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**Introduction:**

**At present there is no clinically effective treatment for injuries or pathological processes that disrupt the continuity of axons in the mature central nervous system including spinal cord injuries. However, a number of studies suggest that a tremendous potential exists for developing extracellular matrix based therapies specific for individual cell types utilizing biomaterials or hydrogel components for cell attachment. In particular, cell specific neuronal extracellular matrix in the form of bridging substrates have been shown to support at least some level of axonal regeneration across the lesion site, but display a limited capacity for directing axons toward their targets. Most cells in tissues contact an extracellular matrix on at least one surface. These complex mixtures of interacting proteins provide structural support and biological signals that regulate cell differentiation and maintenance. The aim of this study was to develop a novel neuronal cell extracellular matrix which would allow the following specific neuronal cell populations such as hippocampal, cerebral cortex and dentate gyrus to grow and make connections with other neurons in culture. The current neuronal cell culture extracellular matrix increased neuronal survival rates by 85% and cells were able to be maintained in culture conditions up to 6 passages when compared to poly-lysine D and Laminin coated tissue culture flasks.**

**Method:**

Three groups of five sets of neuronal primary cell cultures of hippocampal, cerebral and dentate gyrus were made and maintained from the human, rat and mice species to compare the effectiveness of the present neuronal extracellular matrix with the traditional coated matrix such as Ploy-lysine D, laminin and uncoated tissue culture flasks in this study. Group A consisted of five sets of hippocampal neuronal cell culture from each species seeded and grown on Celprogen's neuronal extracellular matrix specific to the neuronal cell type. Group B consisted of five sets of cerebral cortex. Neuronal cell culture from each species seeded and grown on Celprogen's neuronal extracellular matrix specific to the neuronal cell type. Finally Group C consisted of five sets of dentate gyrus cell culture from each species seeded and grown on Celprogen's neuronal extracellular matrix specific to the neuronal cell type. At each passage up to 6 passages immunological probes markers for oligodendrocytes, astrocytes and neurons were determined throughout the entire study. Cell cultures were maintained in Celprogen's Neuronal Cell Culture Complete Media system and were passaged according to Celprogen's instructions for each specific cell type. Neuronal Cell viability studies were performed with Almar Blue according to manufacturer's instructions.

**Results:**

The cell cultures were maintained in their specific neuronal extracellular matrix and the immunological studies for the presence of neuronal markers were performed as indicated table 1. The viability assay results are tabulated together with the highest cell passage number attainable per individual extracellular matrix in table 1. This neuronal cell culture matrix allowed subcloning of only neuronal cells from a subpopulation of neuronal cell cultures. The neuronal cell cultures in Celprogen's neuronal cell extracellular matrix demonstrated 90% pure neuronal cells as determined by immunological neuronal markers, with only 5 -10 % astrocyte population in cell culture prior to first passage and after the first passage neuronal cell populations were maintained at 95% neurons with 2 – 5% astrocytes in cell culture. The neuronal cell population demonstrated the following markers up to 6 passages neurofliament and MAP-2, as indicated in table 1. The neuronal cell cultures showed the presence of MBP a marker for Oligodendrocytes in cell culture and were selectively removed from the neuronal cell culture to obtain 90% neuronal cell density in culture in Celprogen's Neuronal Growth Media.

Table 1: The table indicates the effectiveness of the various extracellular matrix coated T25 flasks and the neuronal cell culture viability as indicated by Almar Blue cell based assay

| Group | Neuronal Cell Culture | Cell Passage number | Tissue Culture Flasks coated Matrix | % Survival (viability) | Neuronal markers |
|---|---|---|---|---|---|
| A | Human Hippocampal (5) | 6 | Neuronal Matrix | 85% | NF |
| | Mouse Hippocampal (5) | 4 | Laminin | 70% | MAP-2 |
| | Rat Hippocampal (5) | 3 | PolyLysine D | 60% | |
| | | 1 | Uncoated | 25% | |
| B | Human Cerebral Cortex (5) | 5 | Neuronal Matrix | 83% | NF |
| | Mouse Cerebral Cortex (5) | 3 | Laminin | 65% | MAP-2 |
| | Rat Cerebral Cortex (5) | 2 | PolyLysine D | 58% | |
| | | 1 | Uncoated | 15% | |
| C | Human Dentate Gyrus (5) | 6 | Neuronal Matrix | 85% | NF |
| | Mouse Dentate Gyrus (5) | 4 | Laminin | 70% | MAP-2 |
| | Rat Dentate Gyrus (5 | 3 | PolyLysine D | 60% | |
| | | 1 | Uncoated | 17% | |

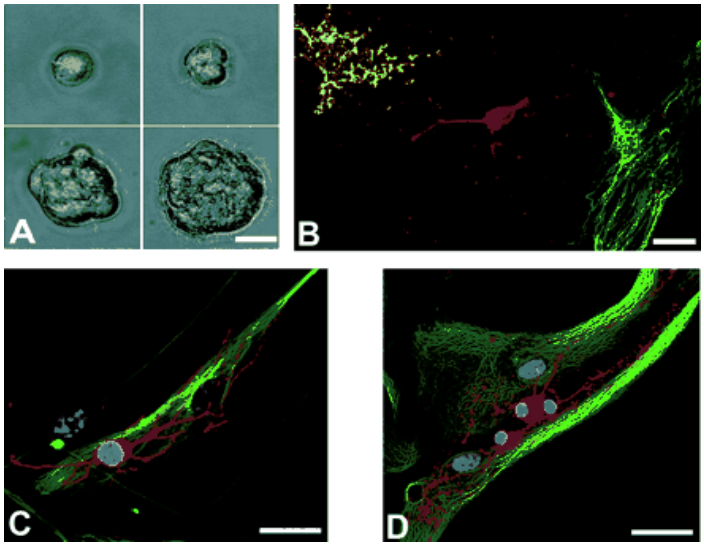


Figure 1: (**A**) The generation of neurospheres in celprogen's neuronal matrix. (**B–D**) Upon differentiation of neurospheres, cells positive for astrocytic (GFAP, green), neuronal [DCX, (**B**) red; MAP-2, C red] and oligodendrocytic markers [O4, B yellow; RIP, (**D**) red] develop. DNA is stained with TO-PRO (blue). Scale bars: 15 µm upper left + right, 25 µm lower left, 40 µm lower right (**A**); 20 µm (**B** + **C**); 40 µm (**D**).

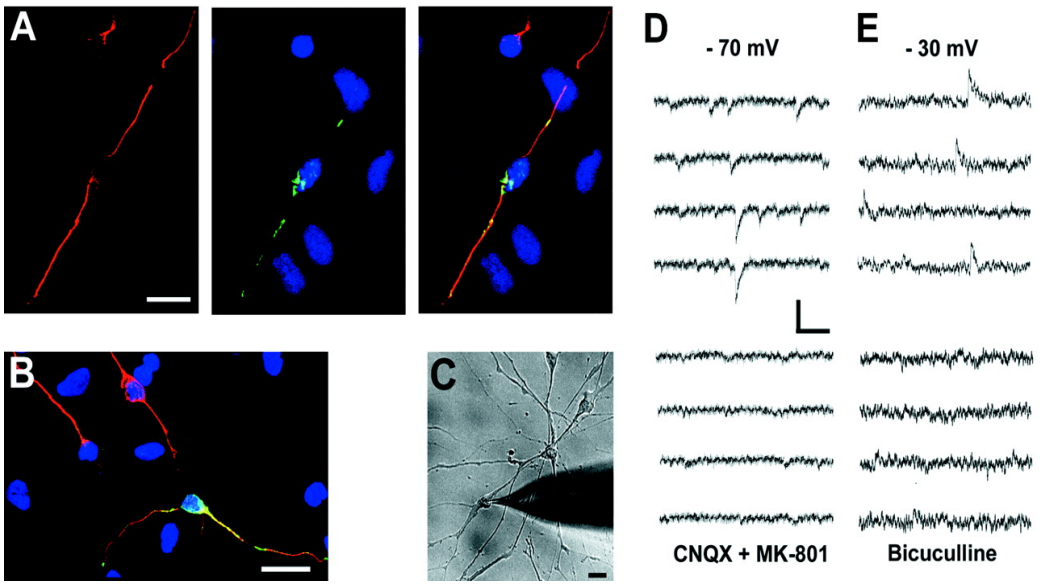


Figure 2: (**A**) Immunocytochemical detection of VGlut-1 (green) in a neuronal cells grown on Celprogen's neuronal extracellular matrix were co-stained with the neuronal marker ß-III-tubulin (red), indicating a glutamatergic neuron. DNA is stained with TO-PRO (blue). (**B**) Co-staining of GAD-65 (green) and ß-III-tubulin (red), indicating a GABAergic neuron. DNA is stained with TO-PRO (blue). (**D–E**) Patch-clamp recordings in neuron positive cells (**C**) lying in a network revealed spontaneous synaptic currents. At a holding potential of –70 mV, the inward currents were blocked by the glutamate-receptor antagonists CNQX (AMPA/kainate) and MK-801 (NMDA) (**D**), indicating glutamate-mediated synaptic communication. In another cell at a holding potential of –30 mV, outward currents were blocked by the $GABA_A$-antagonist bicuculline (**E**), indicating GABAergic synaptic transmission. Scale bars: 20 µm (**A**–**C**); 20 pA and 50 ms (**D**); 15 pA and 200 ms (**E**).

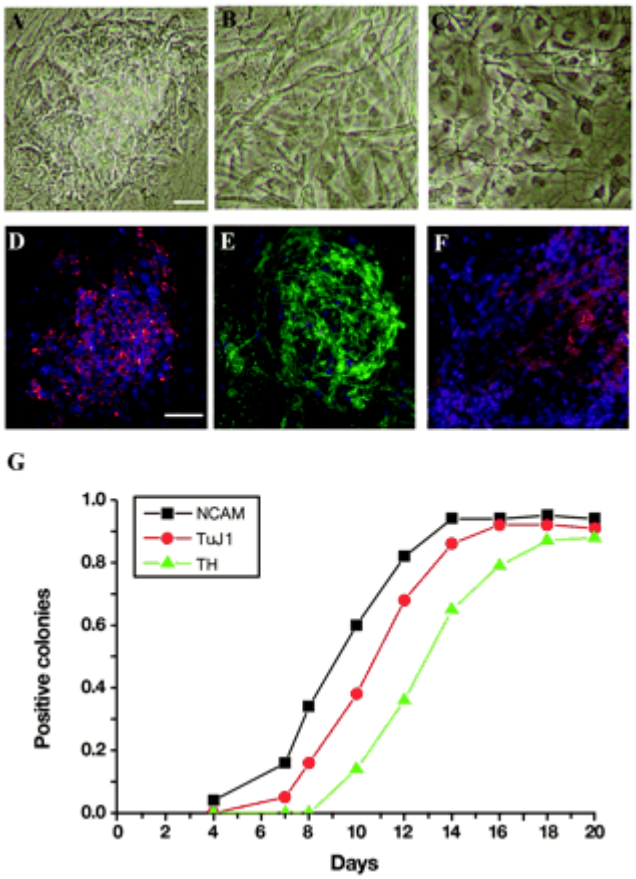


Figure 3: Cerebral Cortical Neural in cell culture in Celprogen's neuronal extracellular matrix. (A–C): Phase-contrast photographs Cerebral Cortical neurons during neural differentiation. (A): A colony with an outgrowth of elongated cells after 6 days in culture. (B): Neurite formation and typical bipolar cellular morphology at day 10. (C): By day 14, cells that migrated out of the colonies displayed bipolar or multipolar morphologies, with extensive development of fine neurites. (D–F): Immunostaining of human Cerebral Cortical Neuronal colonies after 12 days of culture on a Celprogen's neuronal extracellular matrix. (D): Nestin. (E): Neural cell adhesion molecule. (F): TuJ1. Antibody staining is in red (Nestin and TuJ1) or green (NCAM), whereas nuclear 4', 6'-diamidino-2-phenylindole staining is in blue. (G): Time course of the appearance of neural markers during culture of human Cerebral Cortical Neuronal Cells. The percentage of colonies positive for each marker is shown as a function of the number of days in culture on Celprogen's Extracellular neuronal matrix. NCAM first appeared on day 5, whereas TuJ1 appeared on day 8. TH-positive cells were first seen after 10 days. The scale bar is 20 µm. Abbreviations: NCAM, neural cell adhesion molecule; TH, tyrosine hydroxylase; TuJ1, neuron-specific class III beta tubulin.

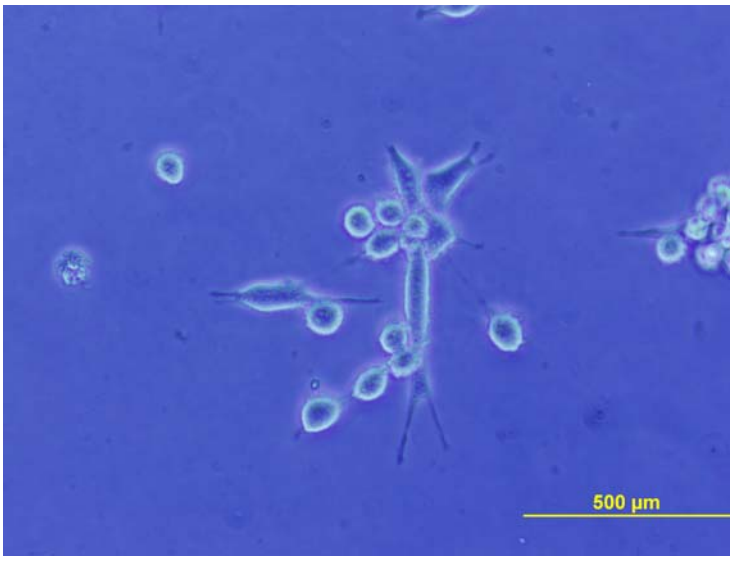


Figure 4: Rat Hippocampal neuronal cell culture grown in Laminin coated tissue culture flask, 24 hours in culture.

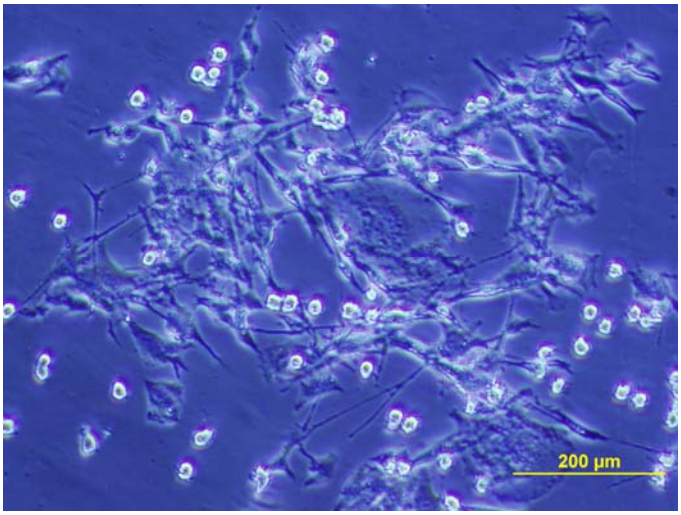


Figure 5:  Hippocampal neuronal cell culture grown in Celprogen's Neuronal extracellular matrix coated tissue culture flask, 24 hours in culture.

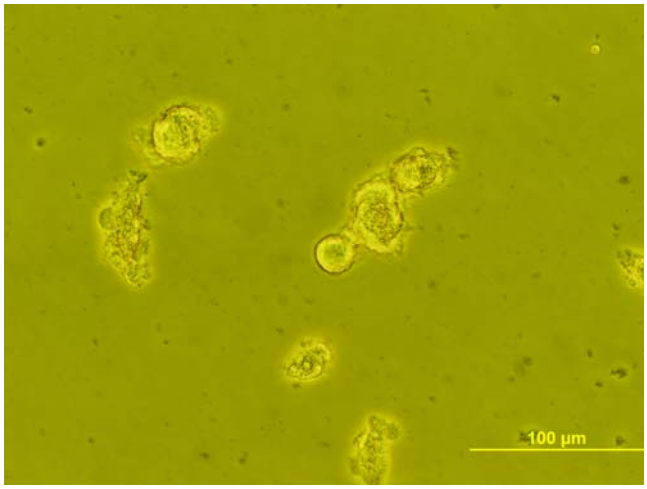


Figure 6: Hippocampal neuronal cell culture grown in uncoated tissue culture flask, 24 hours in culture.

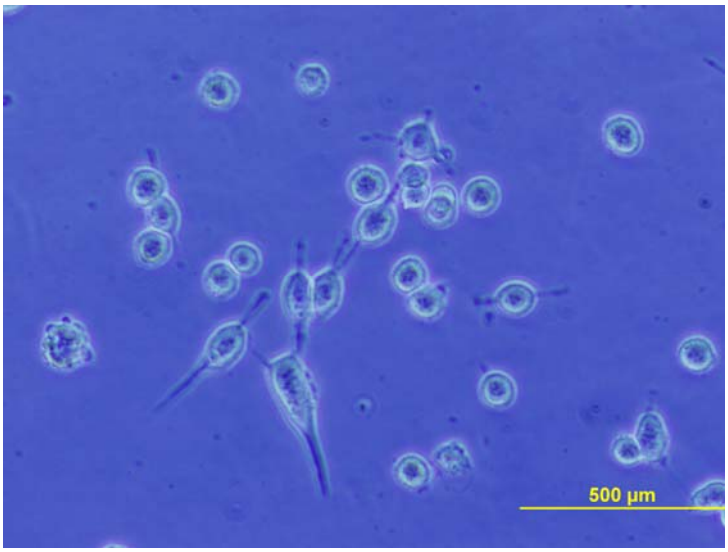


Figure 7: Hippocampal neuronal cell culture grown in PolyLysine D coated tissue culture flask, 24 hours in culture.

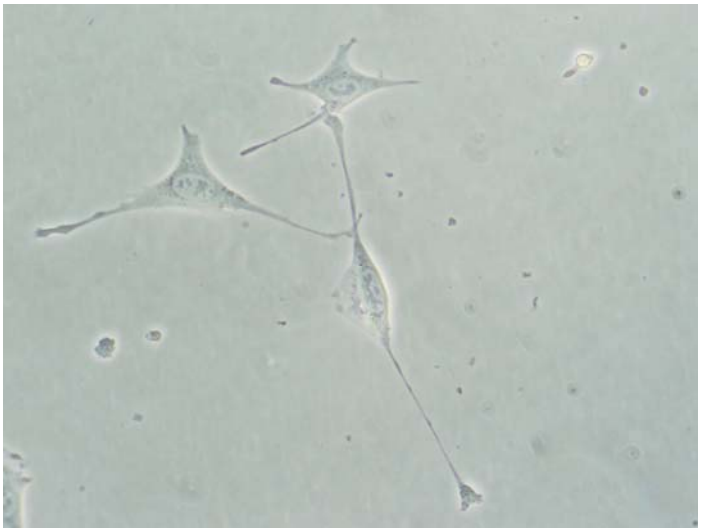

Figure 8: Human Cerebral Cortical neuronal cell culture grown in Celprogen's neuronal extracellular matrix coated tissue culture flask, 7 days in culture indicating neuronal connections of three neurons in culture.

**CONCLUSIONS:**

The present study demonstrated that primary neuronal cells can be maintained in tissue culture for 6 passages with better survival rates in Celprogen's Neuronal Extracellular matrix and neuronal growth media. As indicated in figure 2 the presence of neuronal transmitters in this neuronal culture system can be evaluated. The current Celprogen's neuronal culture system which includes the extracellular matrix and the complete growth media continuously circulating 10 ml tissue culture vessel would enable greater success in the research and development of neuronal transplants for spinal cord injuries and or pathological neuronal conditions / processes.

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