

Extracellular Matrix Proteins Influence Cell Morphology and Function in Rat Neural Cultures

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Abstract

The interaction between extracellular matrix (ECM) and neural cells is important in cell differentiation and morphogenesis of the nervous system. However, conditions for culture of neuronal cells have not been optimized for ECM, and poly-lysine often prevails as the substrate of choice. Quantitative Ca⁺⁺ fluorimetry was used to screen ECM proteins singly and in combination to determine culture conditions resulting in optimal morphology and function of rat cortical cells. A fluid handling robot was used to ensure uniformity in cell plating and reagent dispensing. Agonist responses in cells from single isolations plated on laminin followed by human fibronectin (LM/HFN) were significantly enhanced when compared with cells cultured on LM or HFN alone, as well as on poly-lysine or uncoated tissue culture plastic. In addition, differences in morphology between cultures grown on the various substrates were observed. Pyramidal neurons exhibited long interconnecting processes with growth cones on LM/HFN. Neurites in LM or HFN cultures were significantly shorter and cell bodies formed aggregates on LM. Elongated neurites were characteristic of cells grown on poly-lysine. Preliminary studies using time lapse confocal microscopy with a fluorescent Ca⁺⁺ dye (Fluo3AM) indicated that glutamate receptor function also varies in cells cultured on different ECM substrates, correlating with immunological quantitation of the glutamate receptor (GluR1). Neural cells grown on LM/HFN displayed improved cell morphology and receptor function. These studies indicate that ECM is an important component to consider in obtaining optimal neural cell cultures.

Introduction

HFN, LM, and other ECM proteins play important roles in cell surface interactions (Yamada, 1991). HFN mediates cell adhesion, embryonic cell migration and wound healing, while LM can promote processes as diverse as axonal outgrowth, maintenance of polarized epithelial cells and metastasis. Specialized domains of these molecules or peptide recognition sequences bind specific cell surface receptors, collagens, proteoglycans, or other ECMs. Adhesion molecules in the nervous system are involved in axon elongation and mediate interactions between growth cones and substrates. In addition to HFN and LM, the glycoproteins N-cadherin, NCAM, and other members of the immunoglobulin superfamily play regulatory roles in neuronal function.

Purpose: To optimize cortical cultures by quantifying interactions of neural cells in culture with ECM proteins.

Fluorimetry on 96-well plates and confocal imaging of coated coverslips were used to measure ECM effects on:

- cell morphology
- expression of glutamate receptors
- cell adhesion
- activity of glutamate receptors
- development of dendrites and axons

Materials and Methods

ECM coatings: Corning® BioCoat™ Cell Environments (Corning Life Sciences) for LM, HFN, PDL, LM/HFN on coated 96-well Tissue Culture Plates Corning Cat. No. 353075 and 22 mm² coated glass coverslips were used to analyze neural cultures from neonatal rat cortex.

Cell culture: Neonatal rat cortex were excised from P0-P1 rat pups, the hippocampus was removed and the cortex was cleaned of all meninges. Cortices were dissociated in a solution containing 100 mM CaCl₂, 50 mM EDTA, 2 mg Lcysteine, 200 U/ml papain (Worthington) for 40 minutes at 37°C. Enzyme activity was stopped with 15 mg trypsin inhibitor, 15 mg bovine serum albumin in 10 ml in complete media [DMEM/F12 (phenol red-free, Life Technologies), 10% fetal bovine serum (Life Technologies), pen/strep solution, 20 mM glucose]. Cells were released by trituration through a 25 ml pipette. Isolated cells were either plated into the 96-well plates or settled onto ECM coated coverslips. A Quadra 96 (Tomtec) fluid handling robot was used to create uniform cultures containing 25-30,000 cells per well in the 96-well plates. Coverslips were plated with densities of 1,000 cells/cm².

Effects of ECM coatings: Morphology of neural cells grown on LM, HFN, PDL, or LM/HFN was studied in a 96-well format using Phase Contrast Interference Microscopy. Cells grown on ECM-coated coverslips were imaged using a Nikon PCM2000 confocal scanning laser microscope. Cells were stained with Fluo3AM and dye was excited at 488 nm with an Argon laser. Images were printed using an Epson 600 color printer. Immunohistochemistry was used to quantify the fluorescence levels of labeled neurons and glial cells in a culture, adhesion to an ECM coating, presence of actin-containing processes as well as dendrites and axons, and expression levels of the glutamate receptor.

Stained with:

Neurons Neurotag - Boehringer Mannheim

Glial Cells anti-GFAP (glial marker) - Boehringer Mannheim

Adhesion anti-FAK (focal adhesion kinase) - Transduction Laboratories

F-Actin Phalloidin - Molecular Probes

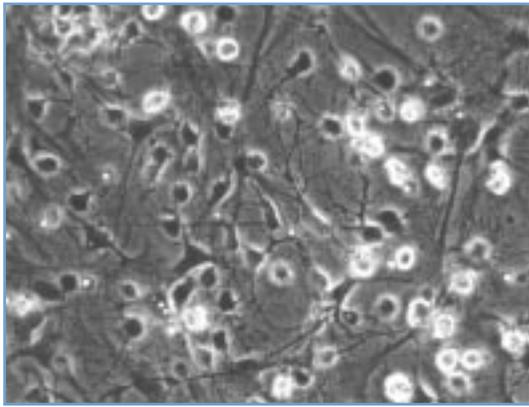
Dendrites Map2 (microtubule associated protein) - Boehringer Mannheim

Axons Tau - Boehringer Mannheim

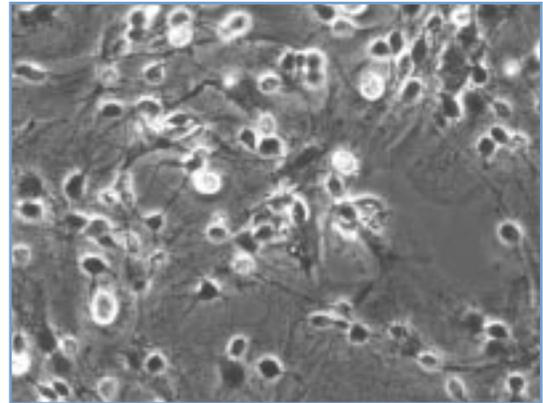
Cells were fixed (5 minutes each) with 30% EtOH/1% acetic acid, 60% EtOH/1% acetic acid and 95% EtOH/1% acetic acid to maintain antigenicity. Cells were permeabilized with TritonX100 (1% for 1 minute) then incubated with the primary antibodies overnight at 4°C except for Neurotag and phalloidin which were incubated for 2 hours and 5 minutes, respectively. A secondary antibody with a direct fluorescent tag was used to label the bound primary antibody. Fluorescence was quantified using a CytoFluor® II fluorimeter (Life Technologies). Immunohistochemical staining of these antibodies was viewed on the Nikon PCM2000 confocal scanning laser microscope. Ca²⁺ imaging and fluorimetry was used to quantitate neurotransmitter receptor activity of cells grown on different ECM coatings. When receptors are bound at the cell surface, there is a resulting

Ca²⁺ spike which can be measured using a Ca²⁺ fluorescent dye, Fluo3AM. Cultures were loaded with Fluo3AM (2 uM) for 40 minutes at 37°C. The 96-well coated plates were rinsed with saline using the robot and were placed on the fluorimeter to obtain a “control” reading. Neurotransmitter solutions (Glutamate, Kainate, GABA, and Acetylcholine, 100 uM) were then added to the wells and a second “agonist” reading was taken. Results were expressed as % Change in Fluorescence. These same Ca²⁺ transients were viewed in time lapse videos using the Nikon PCM2000 confocal microscope. At this level of resolution, individual neurons and glial cells were identified and localized immunohistochemical labeling was recorded.

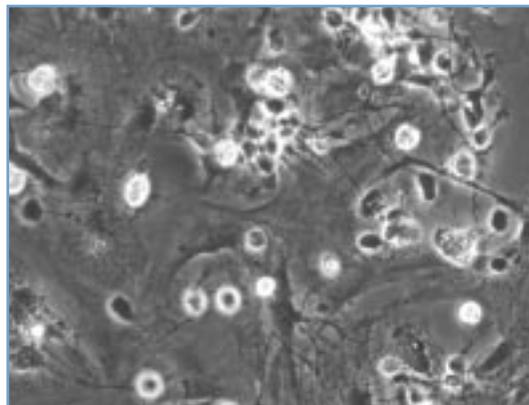
ECM Affects Morphology



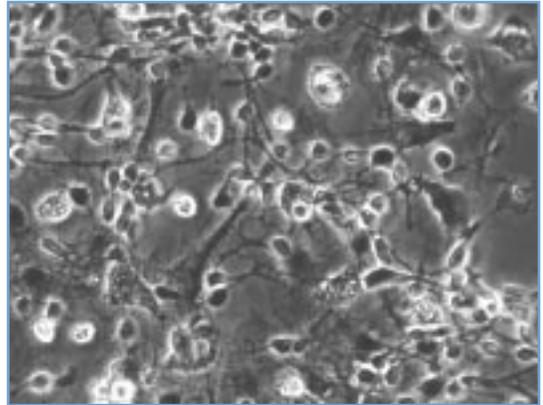
PDL: Neurons highly branched with very long processes. Astrocytes show similar process elongation.



LM: Many multipolar and bipolar neurons with processes which have secondary branches starting on peripheral dendrites. Astrocytes flattened and spread.



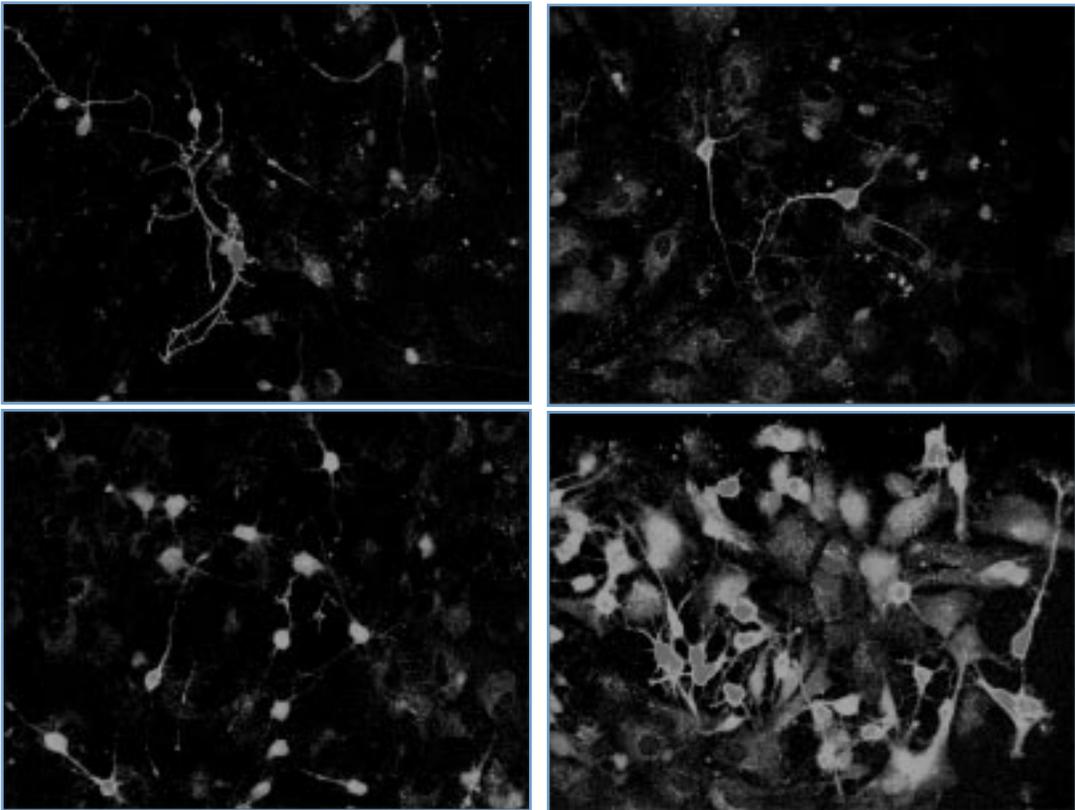
HFN: More types of neurons: multipolar, bipolar, and pyramidal. Fewer dendrites per cell with branches starting closer to the cell body. Astrocytes have spread.



LMIHFN: Neurons have thicker dendrites, multiple spines, secondary branches, and display growth cones. All neuronal types present. Networks are established. Astrocytes have spread.

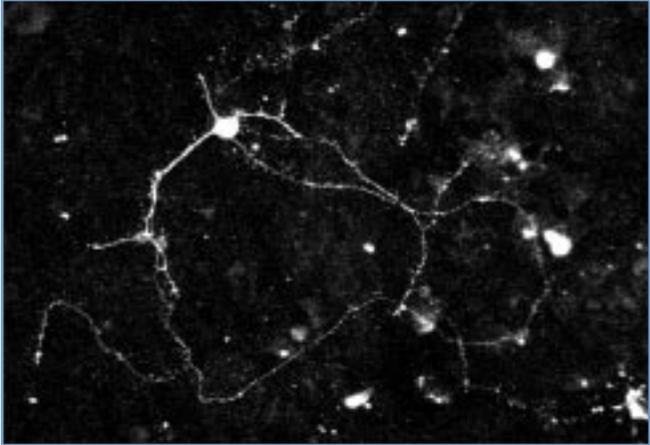
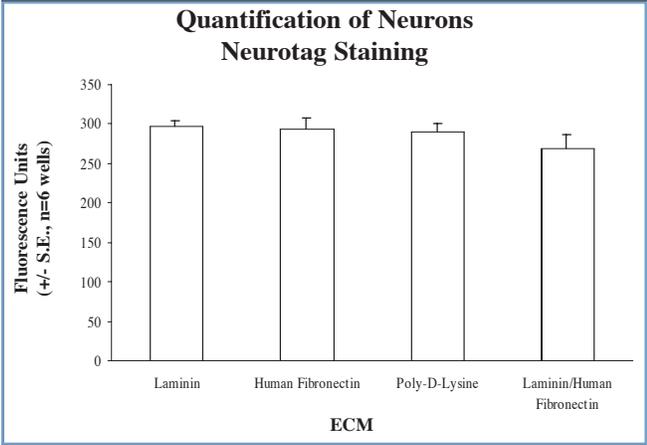
Phase contrast interference micrographs of neural cultures (5 days *in vitro*) grown on PDL, LM, HFN, and LMIHFN ECM proteins.

Confocal Images on Different ECMs

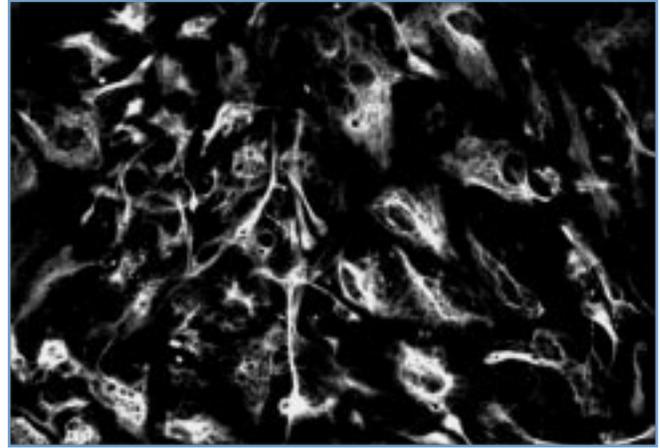
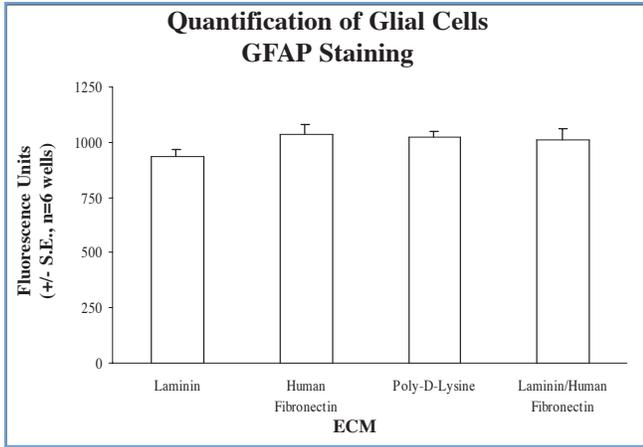


Confocal Images on Different ECMs Show the Morphology In greater Detail. Neurons on Pdl Coating Show very Long Processes With Extensive Branching (Top). Lm Coating Shows Neurons With long Processes With few Secondary Branches (Bottom).

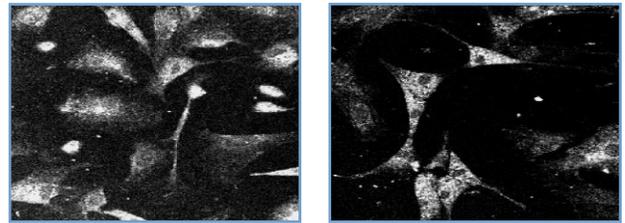
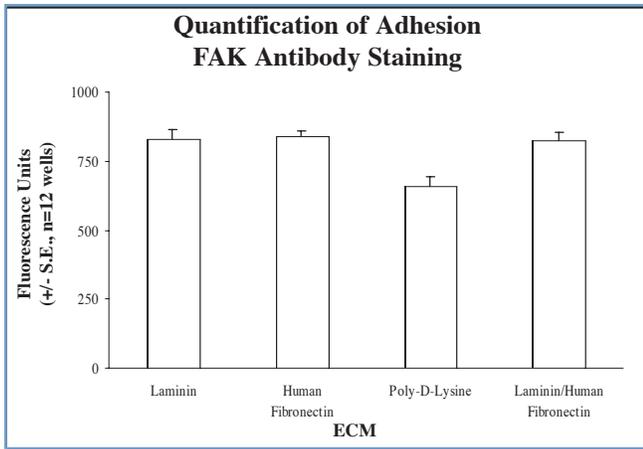
Confocal Images on Different ECMs show Long processes On neurons Grown on hfn (Top). Dendritic Branching Occurs close to Cell body. Neurons grown On Lm/hfn Show very long Extensive Processes with Secondary Branches (Bottom).



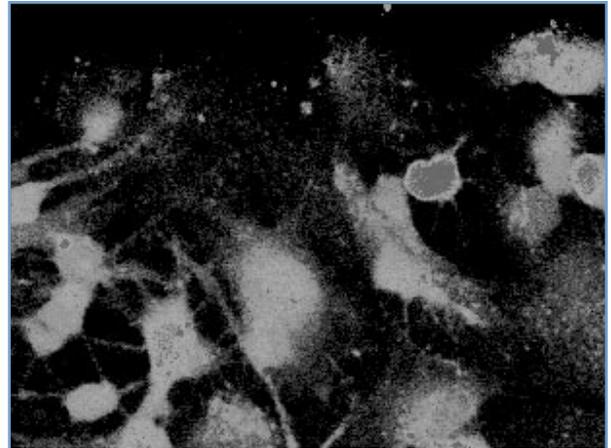
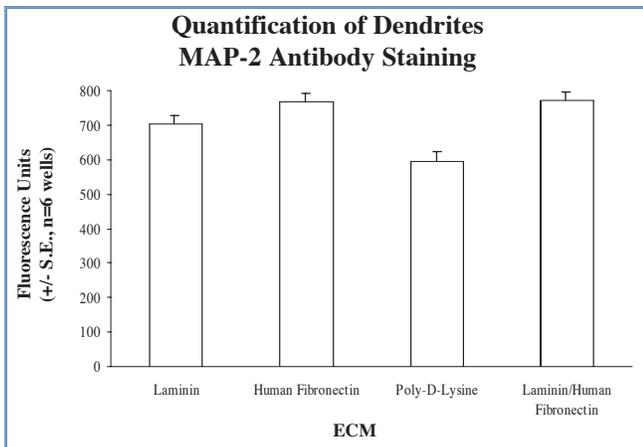
ECM does not affect neuron profile. Similar Neurotag staining on all coatings was measured using fluorimetry (left). Confocal micrograph shows characteristic dendritic branches close to the cell body when neurons are grown on HFN (right).



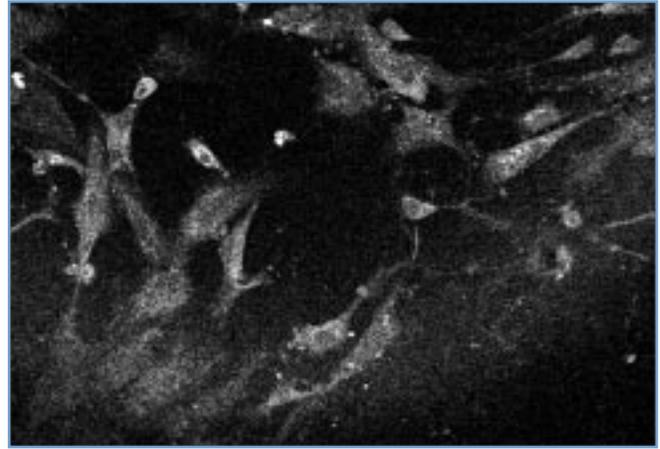
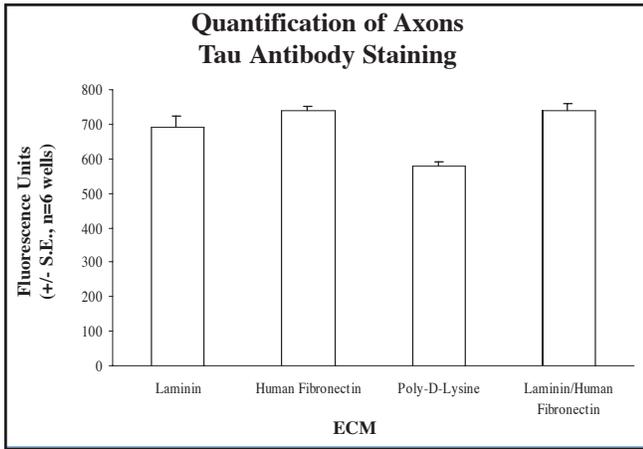
ECM does not affect glial cell profile. All coatings had similar glial profiles as judged by GFAP fluorescence (left). Confocal immunohistochemistry shows the complex morphology of astrocytes grown on HFN (right).



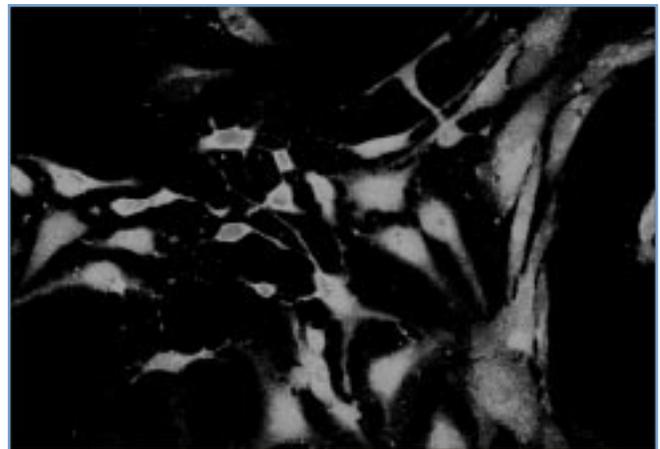
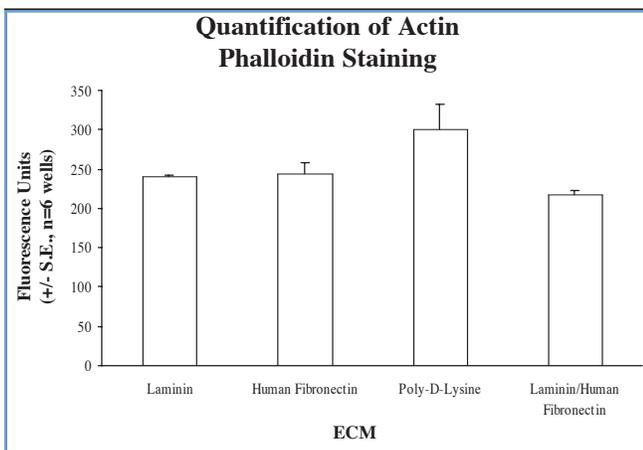
ECM affects cell adhesion. Anti-Focal Adhesion Kinase antibodies localized adhesion plaques in 96-well plates coated with different ECMs. Fluorimetry revealed that coatings containing LM and HFN had greater fluorescence due to FAK (left). PDL was less adhesive. FAK was localized to large plaques in neuronal cell bodies (middle) and to plaques throughout the astrocyte cytoplasm (right).



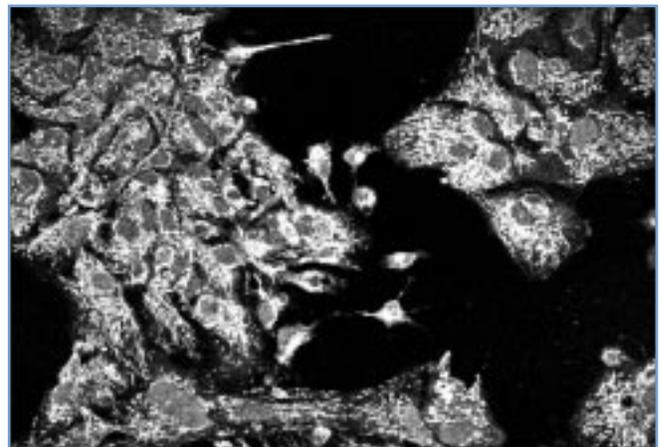
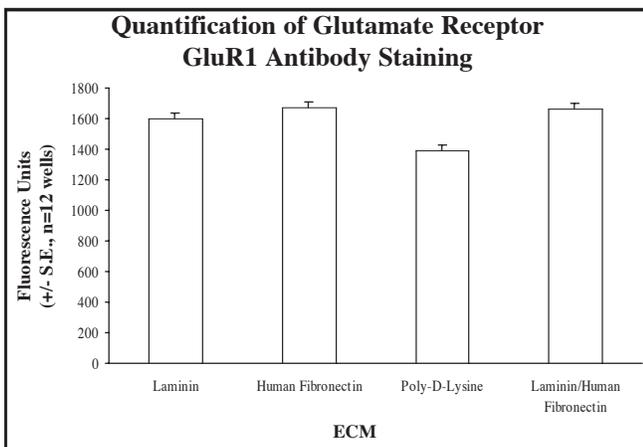
ECM affects dendrite outgrowth. Anti-map2 higher on HFN coatings or combinations shown by fluorimetry (left). There is significantly less MAP2 fluorescence on PDL coatings. Confocal images show MAP2 along dendrites and in neuronal cell bodies (right).



ECM affects axon outgrowth. Anti-tau staining lower on pdl coating seen with fluorimetry (left). There are significantly fewer axons on pdl. Confocal images show positive tau staining in very thin axons and in nerve cell bodies (right).



PDL influences actin polymerization. Phalloidin binding was used to quantitate f-actin in cells grown on ECMs (left). Cells grown on pdl coating exhibited significantly higher fluorescence from f-actin staining quantified with fluorimetry. Confocal images show the filopodia on neurons and lamellipodial veils on astrocytes (right). Morphology is shown for PDL coating, Fluo3AM dye.



ECM affects glutamate receptor expression. Lower glutamate receptor expression on pdl was judged by Fluorimetry using anti-gluR1 receptor antibodies (left). High levels of glutamate receptor seen on cells grown on Lm/hfn (right). Staining extends into small neuronal processes.

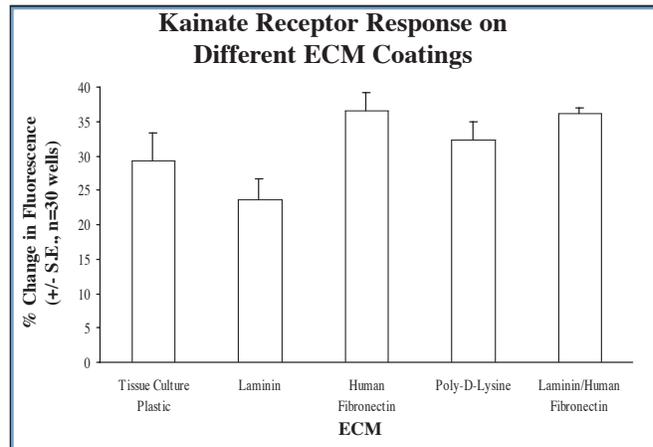
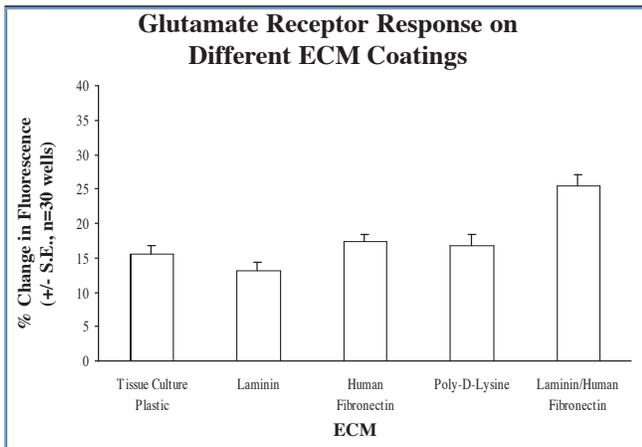
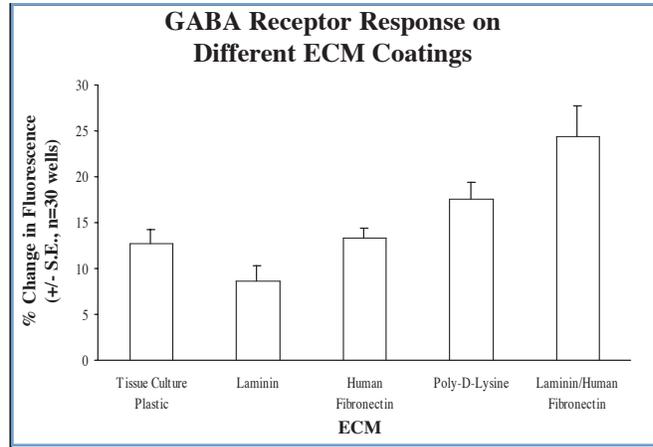
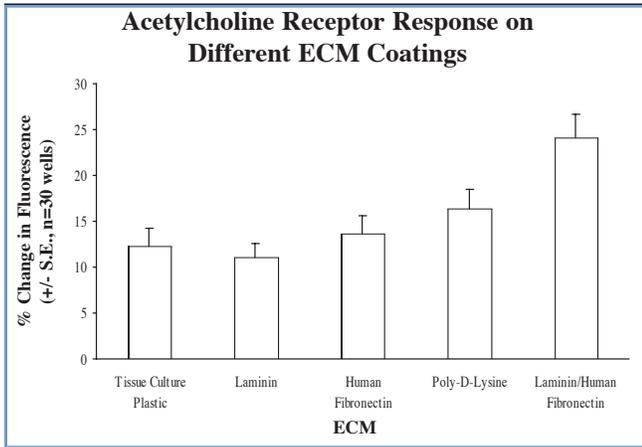
Quantification of Immunofluorescence

Fluorescence units are expressed as Mean +/- S.E. for readings of 8 wells.

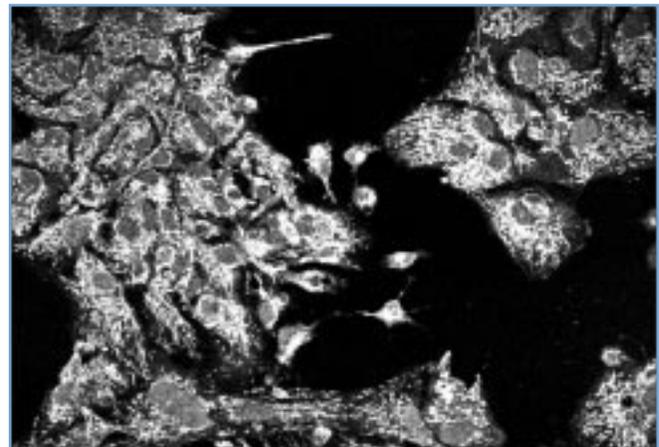
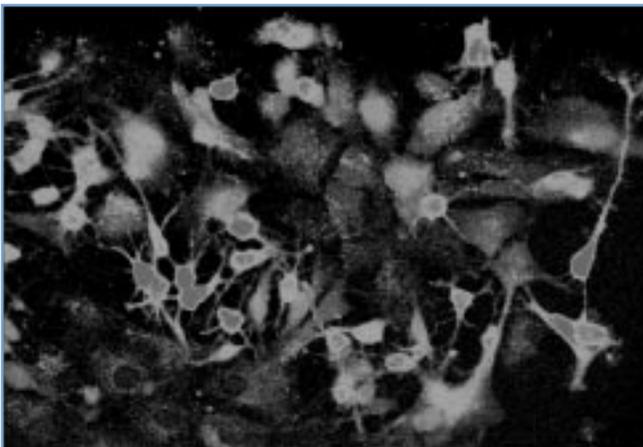
Stain/Fluorescence	ECM Coating							
	LM/HFN	S.E.	HFN	S.E.	LM	S.E.	PDL	S.E.
Anti-GFAP (Glial Cells)	1012.67	46.37	1038.33	40.76	937	32.96	1024.3	26
Neurotag (Neurons)	269	17.03	293	13.65	297.16	7.37	291.17	10.14
Anti-FAK (Focal Adhesion Kinase)	825	31.3	837.84	22.5	826.67	36.4	658	32.8
Anti-MAP2 (Dendrites)	773.33	22.3	767.67	21.6	705	20.77	594	29.57
Anti-Tau (Axons)	740	23.32	739	14.12	693	30.08	577.2	11.8
Phalloidin (F-Actin)	217.6	3.74	243.5	13.85	239.83	3	299.83	32
Anti-GluR1 (Glutamate Receptor)	1665.33	32.24	1669.68	37.97	1598.33	37.13	1349	29.4

Summary of Immunofluorescence Results:

- Cell numbers do not vary significantly on the different ECMs
- FAK staining is lowest on cells grown on PDL
- MAP2 staining is highest on LM/HFN and on HFN and lowest on PDL
- Tau staining is highest on LM/HFN and on HFN and lowest on PDL
- F-Actin staining was highest on PDL
- GluR1 staining is highest on LM/HFN and on HFN and lowest on PDL



ECM affects neurotransmitter receptor function. Fluo3AM fluorescence was used to quantitate neurotransmitter receptor activity of neural cells on different ECMs. Robot-made cultures were used to establish uniform cultures (read day 4 *in vitro*). The greatest responses to glutamate acetylcholine and gaba were observed on *lmlhfn* (left).



Glutamate receptor function on LMIHFN. Time-lapse confocal videos show Ca^{2+} levels increase with activity of the glutamate receptor. Left Panel: saline, Right Panel: 100 μM Glutamate. Ca^{2+} transients increase in neuronal processes as well as cell bodies (Fluorescence=blue<green<yellow<orange).

Conclusion

ECM coatings profoundly affect neural cell morphology as well as function.

1. ECMs affect process outgrowth, branching of neurites, and profile of neural cells. LM and LM/HFN coatings support cells with the most diverse morphologies.
2. ECMs affect adhesion, LM and HFN coatings or combinations have greater adhesion.
3. ECMs affect expression and growth of axons and dendrites. Greatest number of dendrites and axons are seen on HFN and LM/HFN coatings.
4. ECMs affect polymerization of actin filaments. High levels of F-Actin are produced on PDL without concomitant increase in the number of dendrites or axons.
5. ECMs affect expression of the glutamate neurotransmitter receptor. The highest levels of GluR1 are found on LM, HFN, and LM/HFN.
6. ECMs directly affect neurotransmitter receptor activity and function. For glutamate, the greatest activity was seen on LM/HFN. For kainate, cells grown on HFN and LM/HFN have the highest activity levels. For acetylcholine and ABA, cells grown on LM/HFN have substantially higher activity levels.

Corning acquired the BioCoat™ brand.
For information, visit www.corning.com/discoverylabware.

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