Foundation for Research & Technology-Hellas

COST MP1301:
Workshop COST Action MP1301 NEWGEN-New generation biomimetic and customized implants for bone engineering
13-14 October, Sofia, Bulgaria

The effect of micropatterned structures fabricated via ultrashort pulsed laser irradiation on Neuronal Stem Cells

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Contents

- Objectives & Motivation
- Experimental procedures
- Morphological Properties of the micropatterned surfaces
- Cell adhesion, proliferation and alignment of the micropatterned surfaces
- Correlation of the properties
- Conclusions
- Acknowledgments

Objectives & Motivation

Motivation

- **Contact guidance** in terms of topography is an emerging parameter for successful nerve regeneration.
- Schwann Cells (SCs) promote nerve guidance and their directional proliferation affect **neurite outgrowth and alignment for axon guidance**.
- Many groups have used *patterned structures such as combinations of grooves and plateaus* resulting in rather over-simplified topographies for cells under in vivo conditions.
- Hoffman-Kim D group described in vitro a method of replicating the **SCs topography (features and dimensions) as a time-depended setting for directing neurites** (Hoffman-Kim D. et. Al., Acta Biomaterialia, 2013, 9: 7158-7168)
- In clinical **Neural Stem Cells (NSCs)** therapy, the lack of efficient methodologies for large-scale expansion and **controlled differentiation to functional cell types for transplantation**, becomes one of the critical issues for the success of NSC-based therapies (Lin Qi et al., PLOS ONE, March 2013 | Volume 8 | Issue 3 | e59022)

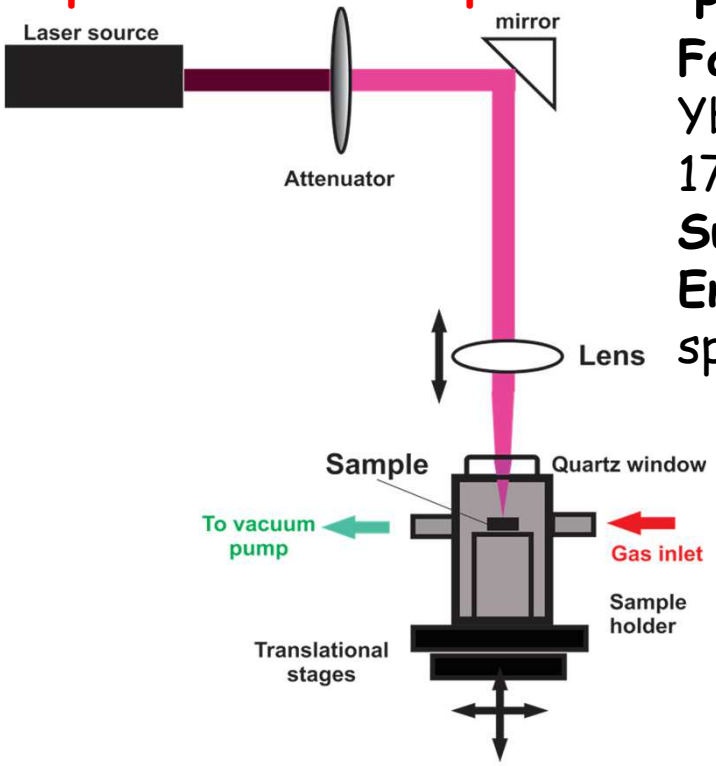
.....To control the topography of patterned Si surfaces via ultrashort pulsed laser irradiation and their PLGA replicas in order to enhance the SCs proliferation and direction and NSCs differentiation for the optimal contact guidance (nerve regeneration strategy)

Contents

Processing of the Micropatterned structures

1.Fabrication of the Si structures via ultrashort pulsed laser (by Chara, Despina and Xristina)

Experimental Set-up



Fabrication Conditions:
Yb:KGW (λ = 1030 nm)
170 fs pulses at a repetition rate of 1 kHz
Substrate = Single crystal silicon (Si)
Environment = Reactive gas SF_6 (650mbar) for spikes and water for ripples/microgrooves

Parameters under investigation

- ✓ Laser Fluence : 0,42J/cm^2, 0.54 J/cm^2 and 0,72J/cm^2 for low, medium and high roughness of spikes
- ✓ Rectangles of 1500μm length and width range of 80 - 400μm
- ✓ Laser Fluence: 10, 50 and 100mW for nanoripples and microgrooves)

2. Replica Fabrication

Negative replica = PDMS (SYLGARD 184, Dow Corning) at 10:1 w:w ratio
Vacuum chamber = For degassing/removal of air bubbles & heating (70° C for 2h)
Positive replica= PLGA (Sigma Aldrich), 65:35 lactide:glycolide, Mw:40000-75000 at solution 10/90 in dichloromethane or acetone
Solvent Evaporation (overnight in –20°C) and then placed in 4° C for 2h

Cells and Methods for the Characterization of the Micropatterned structures

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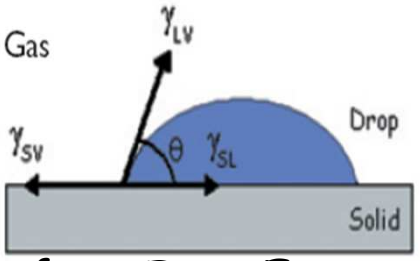
Cell protocols under in-vitro conditions

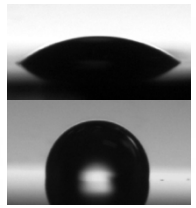
- Murine Neuronal Schwann cell (SCs)– 60000cells per sample
- Neural Stem Cells: Embryonic Cortical 13.5-Passage 4 – 50000 cells per sample
- Different time points (3, 5 and 7 days)

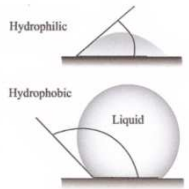
SEM evaluation

- For micropatterned structure morphology, and cellular adhesion, proliferation and alignment
- Standard fixation: Gluterladehyde and consecutive dehydration with 30-100% EtOH and Critical point drying
- Gold coating (thickness 10nm)

Wettability measurements (Contact Angle – CA)







Surface Free Energy

$$\cos\theta = -1 + 2\sqrt{\frac{\gamma_{SV}}{\gamma_{LV}}}e^{-\beta(\gamma_{LV}-\gamma_{SV})^2}$$

More hydrophilic when CA ↓

γLV is the liquid-air interface tension, γSV is the solid-air interface tension. The β an empirical constant equal to 0.0001247 (m2/mJ)2*

Immunostaining protocol (fluorescence micrcoscope)

- **SCs**: Cell viability (nucleus –DAPI), cell adhesion and alignment (Cytoskeleton-Phalloidin/actin)-1:60), **NSCs:** Cell viability (nucleus-TOPRO)-1:10^3, cell proliferation (Nucleus-Ki67 marker-1:200) and NSCs presence (Cytoskeleton – Nestin -1:10^3)

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SEM Images: Micropatterned Si structures (Spikes and Ripples)

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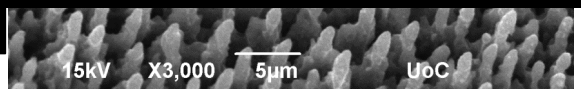
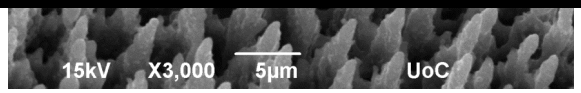
Low roughness -Superhydrophilic Medium - Hydrophilic High - Hydrophilic

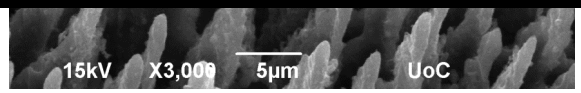
Tilted

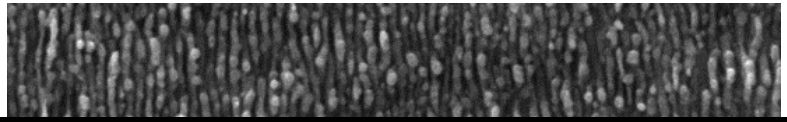
Main Findings - Spikes:

- Morphology of microcones with elliptical cross-section
- Increasing inter-spike distance by increasing the laser fluence (Ranging of roughness – Microscale Topography)
- Controlled geometry and uniformity



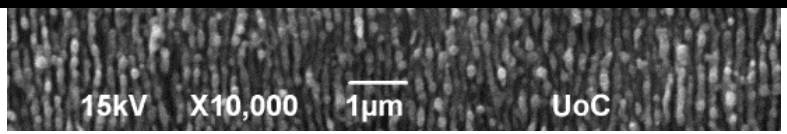






Main Findings - Ripples:

- Morphology of periodic ripples
- Very low laser fluence
- Superhydrophilic
- Nanoscale topography



Main Findings – Spikes on ripples:

- Morphology of microcones (under SF_6 gas) with elliptical cross-section and of periodic ripples (under distilled water)
- Rectangular Lines of length 1500μm and width range of 80-400μm)

SEM Images: Micropatterned Negative (PDMS) and Positive Replicas (PLGA)

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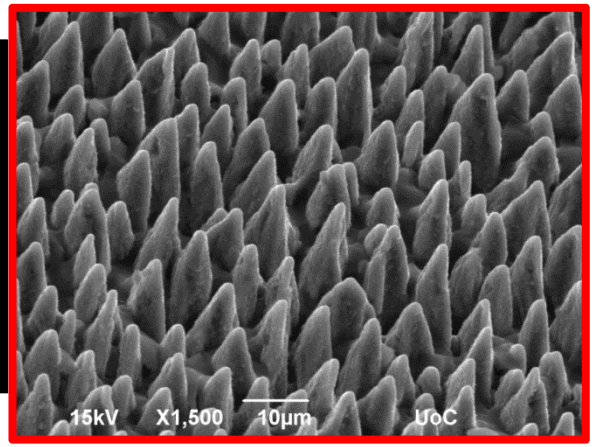
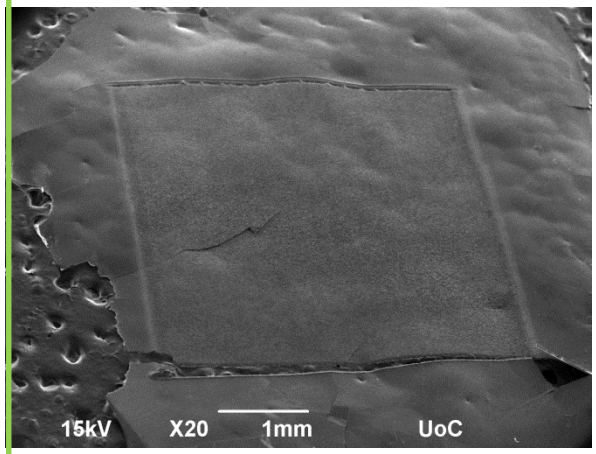
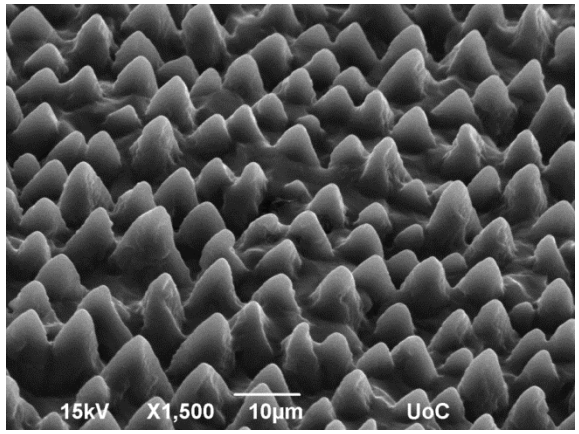
Tilted

Si master

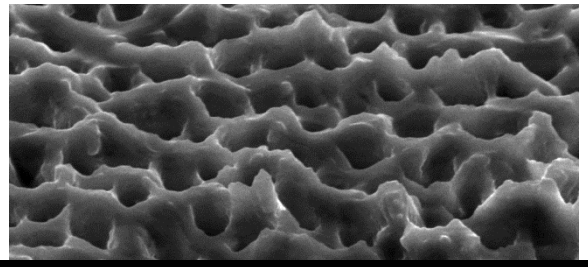


PLGA in Acetone

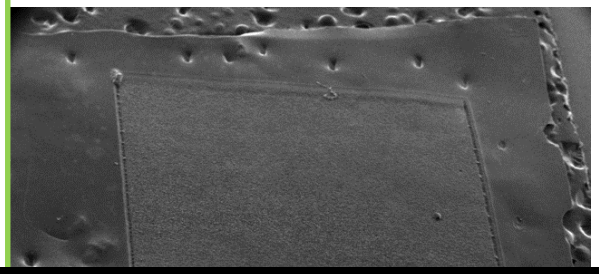


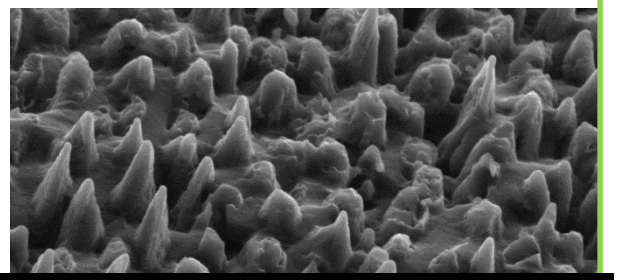


PDMS Negative



PLGA in DCM





Main Findings – Negative and Positive Replicas:

- Successful replication of the PLGA replicas - NEW
- Successful use of the negative PDMS more than one time
- Controlled geometry (as seen from DCM solvent) and pattern regularity (as seen from the Acetone solvent)

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SCs Cell Study:
Adhesion, proliferation and alignment

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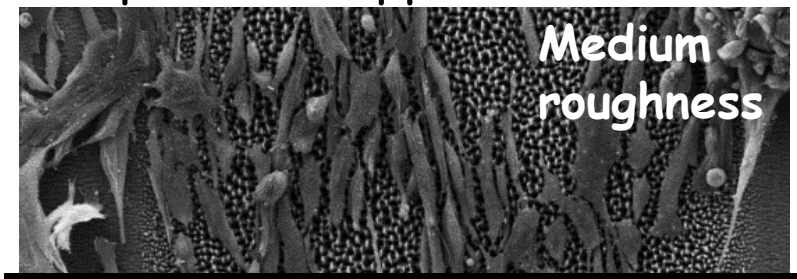
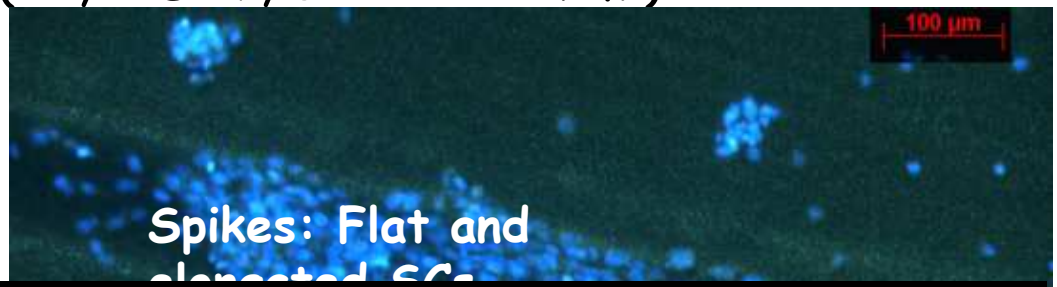
Si Spikes on ripples with SC cells (P5, 3 DIV, $6x10^4$ cells/ml)



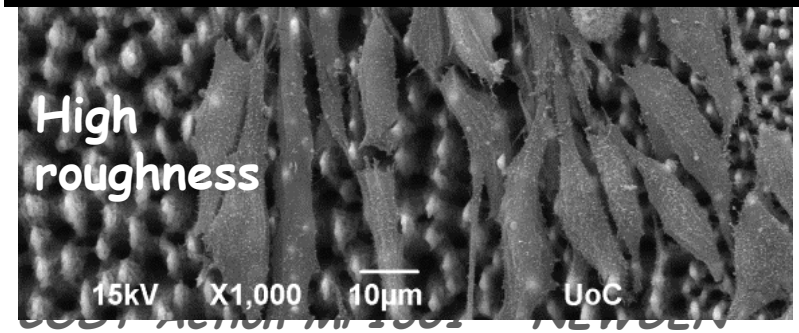


Main Findings

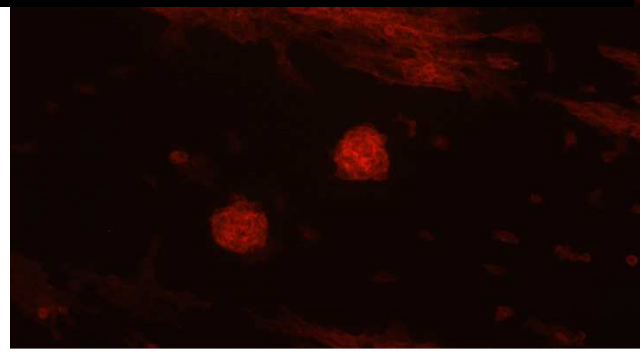
Adhesion: Flat and elongated SCs adhered on the spikes with short and long extensions, formed aggregates on the ripples, and formed SCs networks on the flat Si. Interesting that we can distinguish the top of the spike under the SC soma

Alignment: SCs followed the linearity of the spikes

Proliferation: SCs proliferated and covered the whole surface of the spikes and also communicated between the rectangular lines without adhering on the ripples.



By PhD Candidate,
Despina Angelaki



SCs Study = Adhesion and Viability

PLGA Spikes with SC cells (P6, 3 DIV, $6x10^4$ cells/ml)

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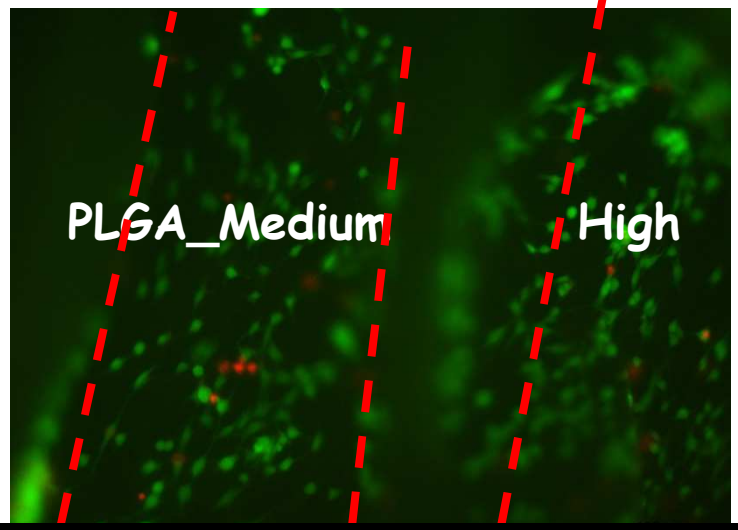
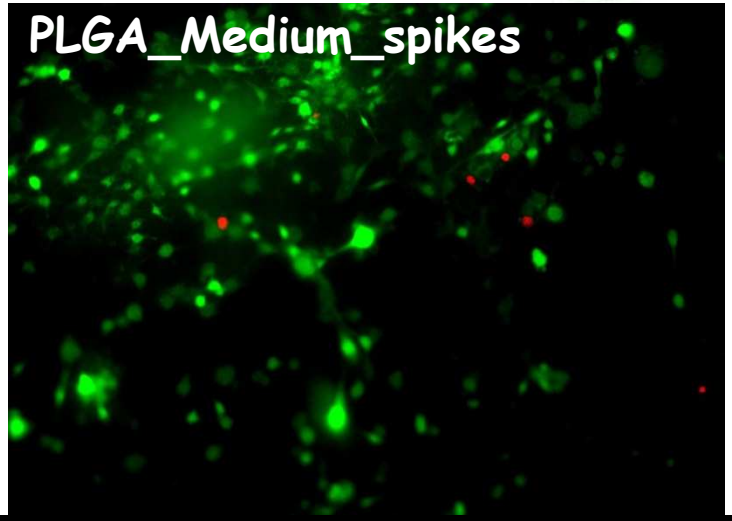
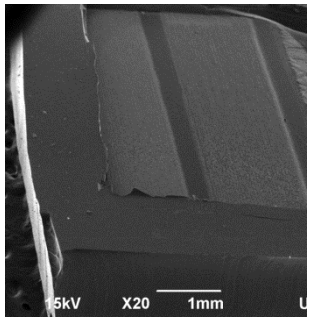


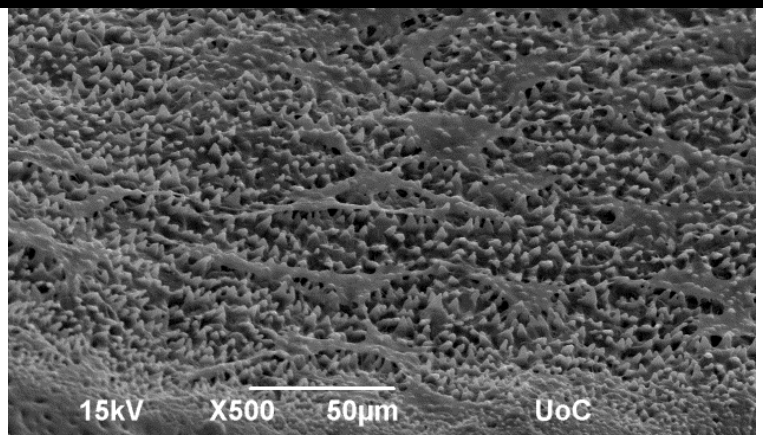


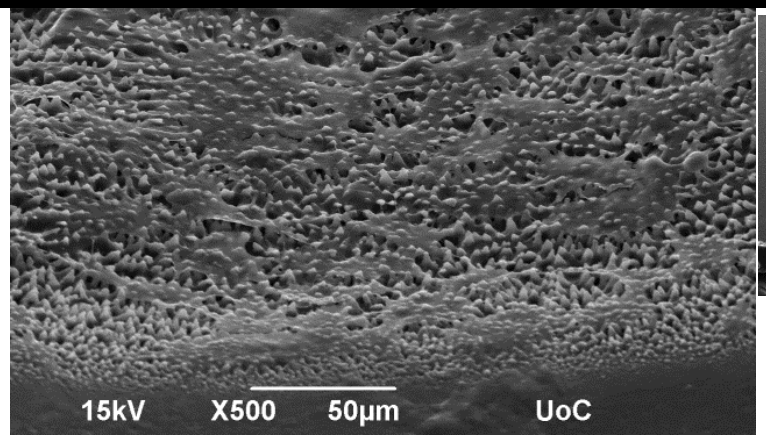


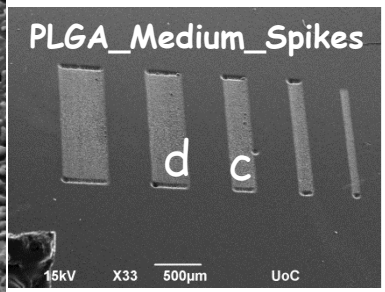
Main Findings

Cell Viability: Presence of SCs on PLGA different patterns

Adhesion: Flat and elongated cells with long and short (on PLGA) processes / extensions

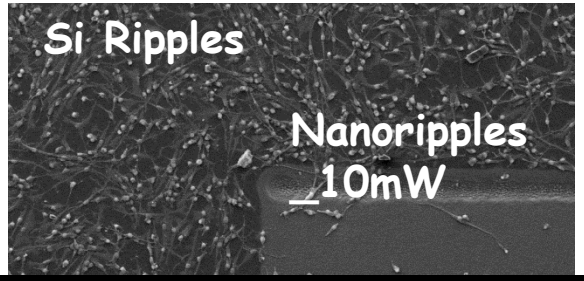


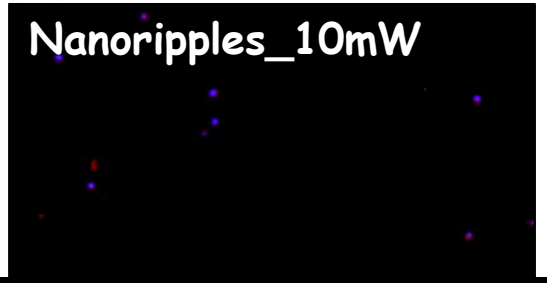


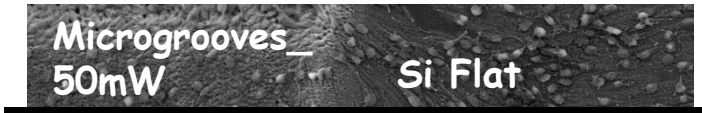


NSCs Study = Viability & Proliferation

NSCs [Cortex E13.5-Passage 4]
7 DIV on Si Ripples/Microgrooves

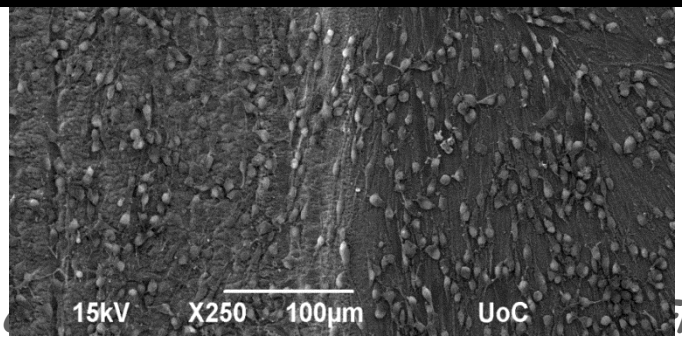


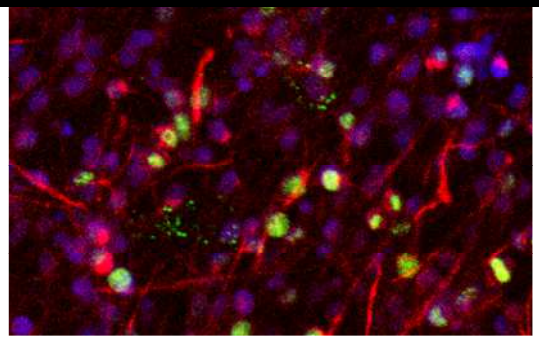


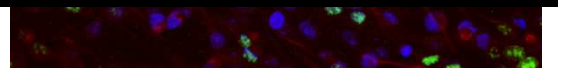


Main Findings

- **Viability:** Presence of NSCs on 3, 5 and 7 DIV on ripples and microgrooves, but they are more on Si flat
- **Adhesion:** Elongated cells with long and short extensions
- **Proliferation:** The microgrooves samples did not exhibit differences in the proliferation and nestin presence for all time periods
- Differentiation trend: Si flat> 100mW microgrooves> 50mW >>>10mW
- Proliferation trend (Ki67): Si flat> 100mW microgrooves & 50mW >>>10mW
- Viability trend (Topro): Si flat> 50mW microgrooves> 100mW>>>10mW







Nestin – NSCs presence

Ki67 – proliferation marker

TOPRO - Nucleus

NSCs Study = Viability & Proliferation

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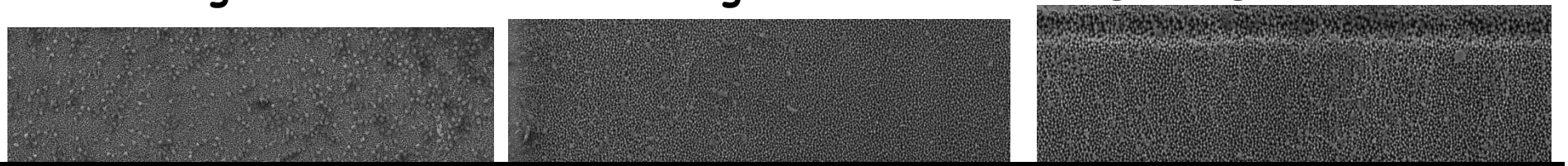
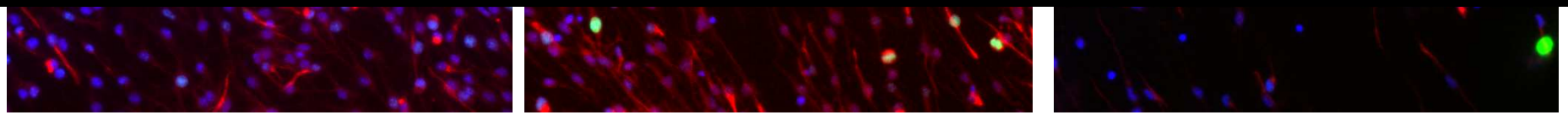
NSCs [Cortex E13.5-Passage 4] **7 DIV on Si Spikes**

Low roughness **Medium roughness** **High roughness**



Main Findings

- **Viability:** Presence of NSCs on 3, 5 and 7 DIV mainly on the flat and patterned Si
- **Adhesion:** Elongated cells with long and short extensions
- **Proliferation:** The medium and low roughness spikes showed higher proliferation and higher NSCs presence (more Nestin) compared to high roughness
- Differentiation trend: Medium roughness > Low > High
- Proliferation trend (Ki67): Medium Roughness > Low> High
- Viability trend (Topro): Low roughness> Medium> High



In collaboration with Dr Kanelina Karali

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Correlation of the Investigated properties under in-vitro conditions

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Material Properties

Surface roughness is altered and depend on:

- Laser irradiation process and the specific laser parameters eg. Laser fluence, environment
- Material physical form and type/composition

Wettability and Surface Free Energy is affected by the :

- Laser irradiation process and other treatments
- Material physical form and composition
- Surface topography and roughness

Cell Type

SCs and NSCs behaved differently:

- **SCs**: flat and elongated on the spikes with combination of long and short extensions, PLGA long extensions, aggregated on ripples, networks on Si flat
- **NSCs**: Elongated with combination of long and short extensions

Material & Cell Interactions

Cell adhesion & proliferation are based on:

- Surface **chemistry** influences the type of integrins (size 8-12nm) recruited and therefore function of the focal contact *
- Surface **roughness** influences the probing of filopodia (size 250-400nm) of the cells*
- Wettability and **Surface free energy**
- Culture conditions (cell type, selected time periods, cell number)

Cell morphology is affected by:

- Surface topography and roughness (eg ripples – cell aggregates while spikes – elongated cells with short and long extensions)*
- Surface chemistry (e.g. PDMS–cells with short extensions and PLGA – cells with long extensions
- Cell adhesion (e.g. focal adhesion quality vs presence of many filopodia)*

*Hoffman-Kim et. Al., Acta Biomaterialia, 2013, 7158-7168, Anselme et.al, Acta Biomaterialia, 2010, 3824-3846

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Conclusions

Laser Irradiation Parameters

1. Laser fluence affects:

- Topography and roughness of the micropatterned structures
- Wettability

2. Environment: SF6 gas forms spikes and water forms ripples and microgrooves

CELLULAR-MICROPATTERNED STRUCTURES INTERACTIONS

3. Depended on the Cell type (SCs and NSCs) and the material type (Si, PLGA)

Motivation

A clever patterned platform navigating neural cells for either optimal contact guidance or not to promote proliferation and differentiation leading to nerve regeneration

proliferation compared to low roughness and Si flat. For NSCs: The medium and low roughness spikes showed higher proliferation and higher NSCs presence (more Nestin) compared to high roughness and Si flat

B. Topography in terms of microgrooves – Nano & Microscale (For NSCs):

- **Adhesion**: Elongated cells with long and short extensions
- **Proliferation**:The microgrooves samples did not exhibit differences in the proliferation and nestin presence for all time periods

Acknowledgments

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- National Funding: Kripis – BIOSYS

THANK YOU
FOR YOUR ATTENTION

PDMS Neg.Repl-Second Run & PLGA_acetone_replica

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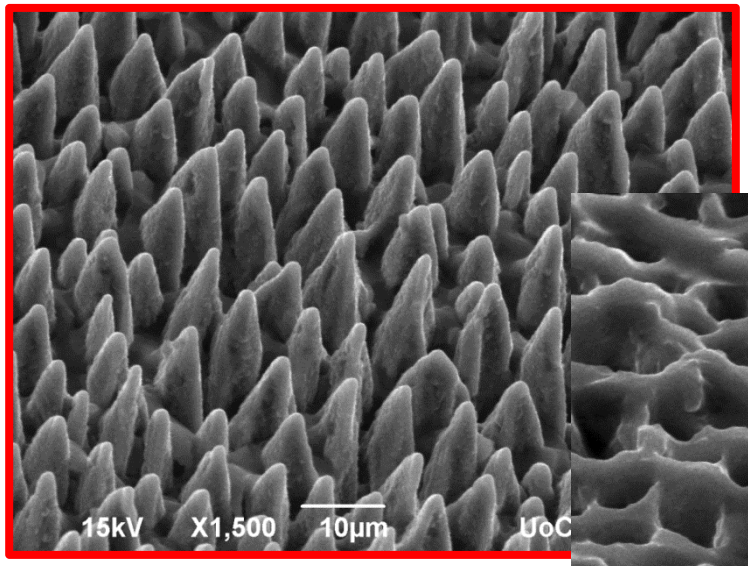
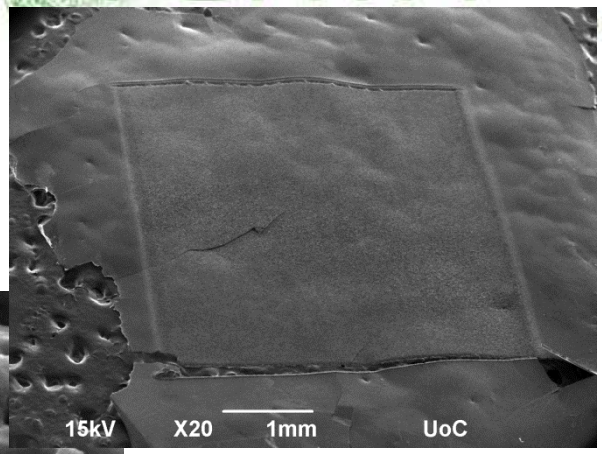
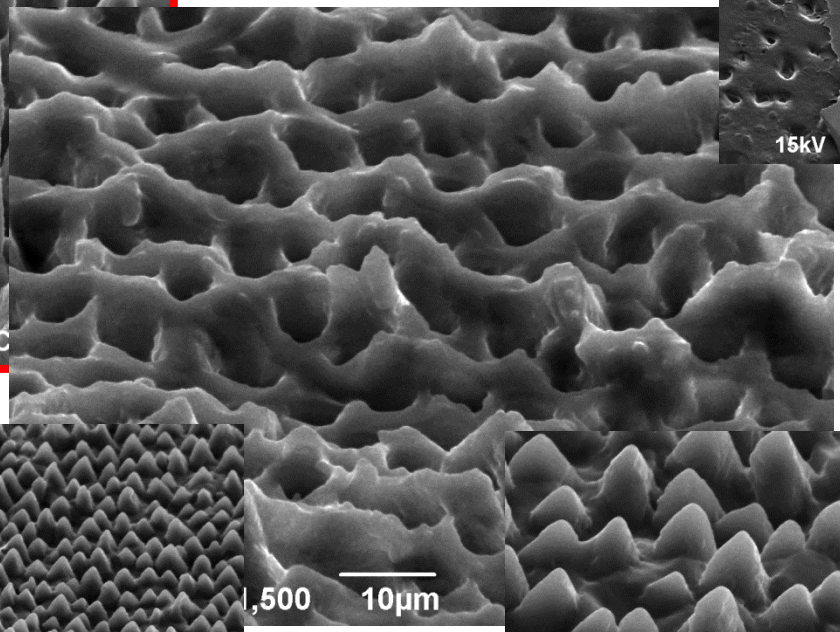
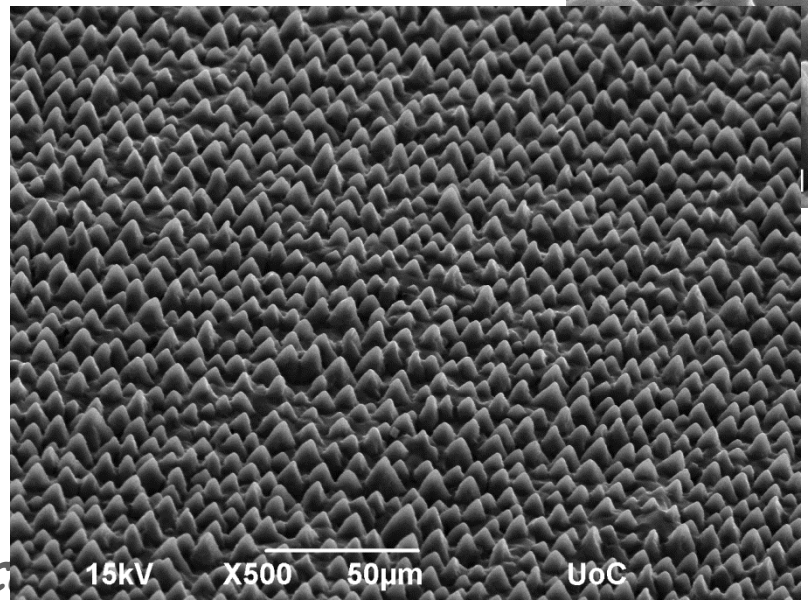
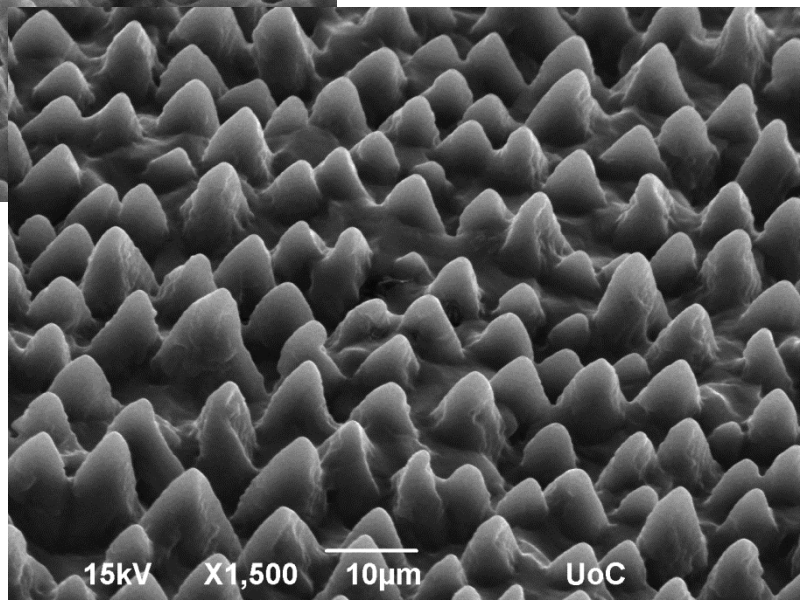


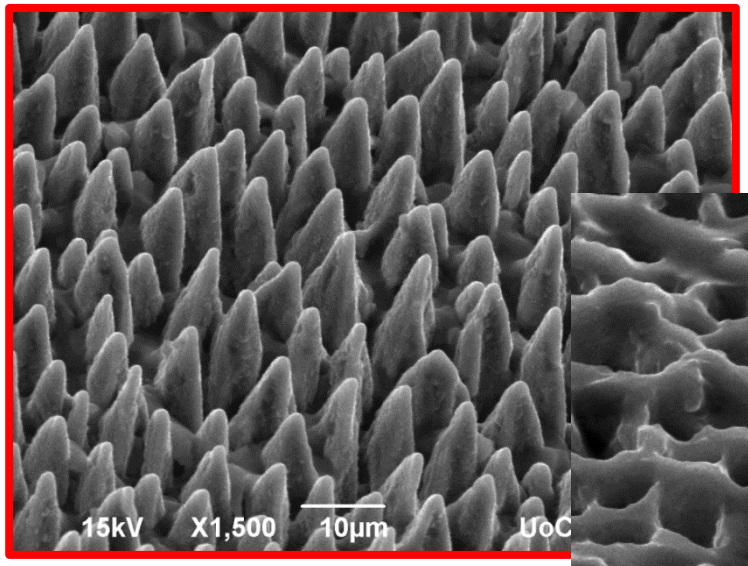


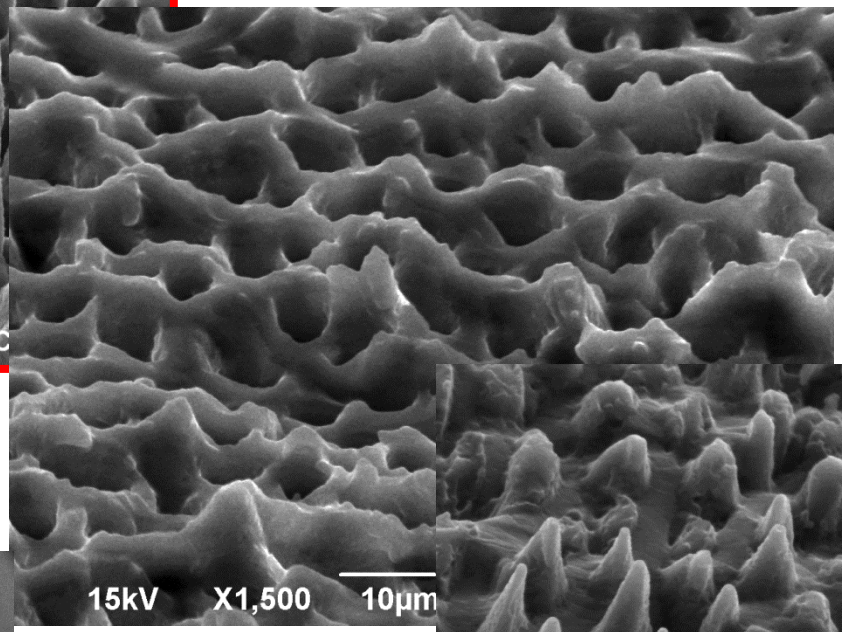


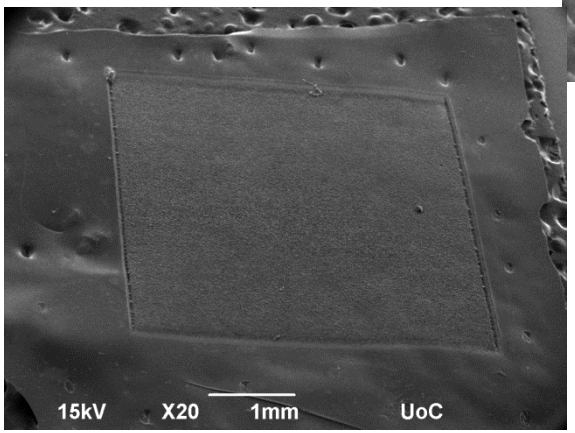


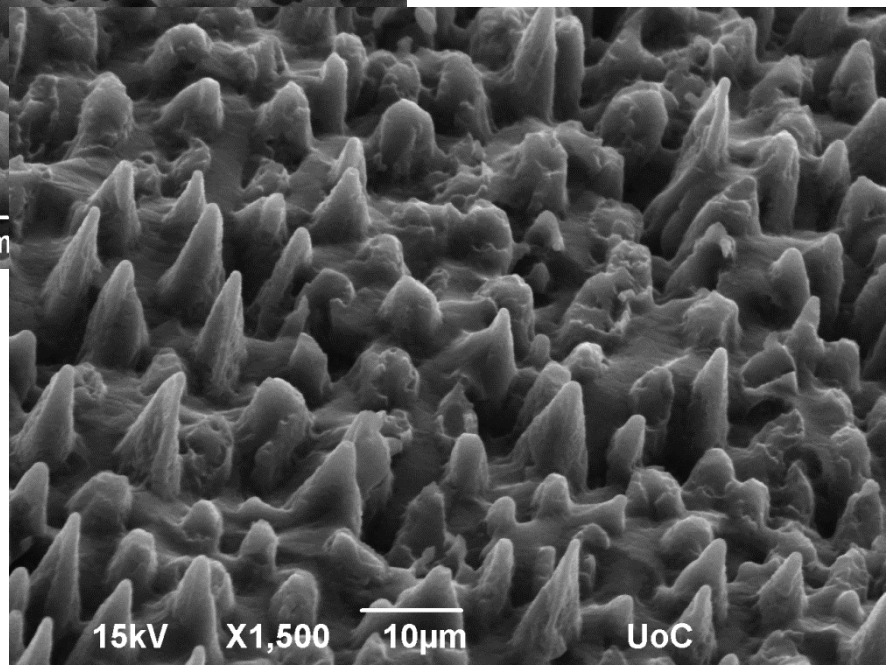


PDMS Neg.Repl-Second Run & PLGA_DCM_replica

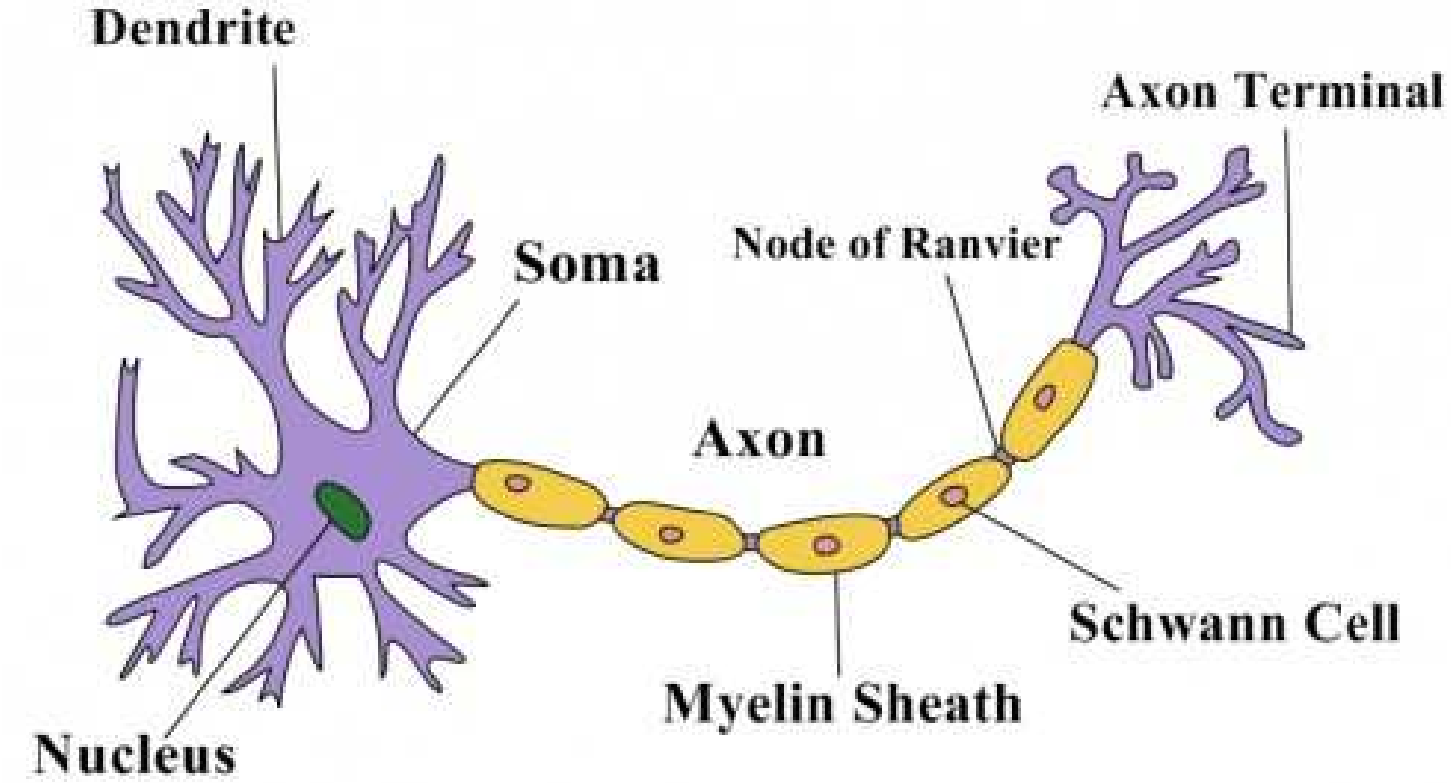








Neuron Structure



http://hubpages.com/hub/Structure-of-a-Neuron