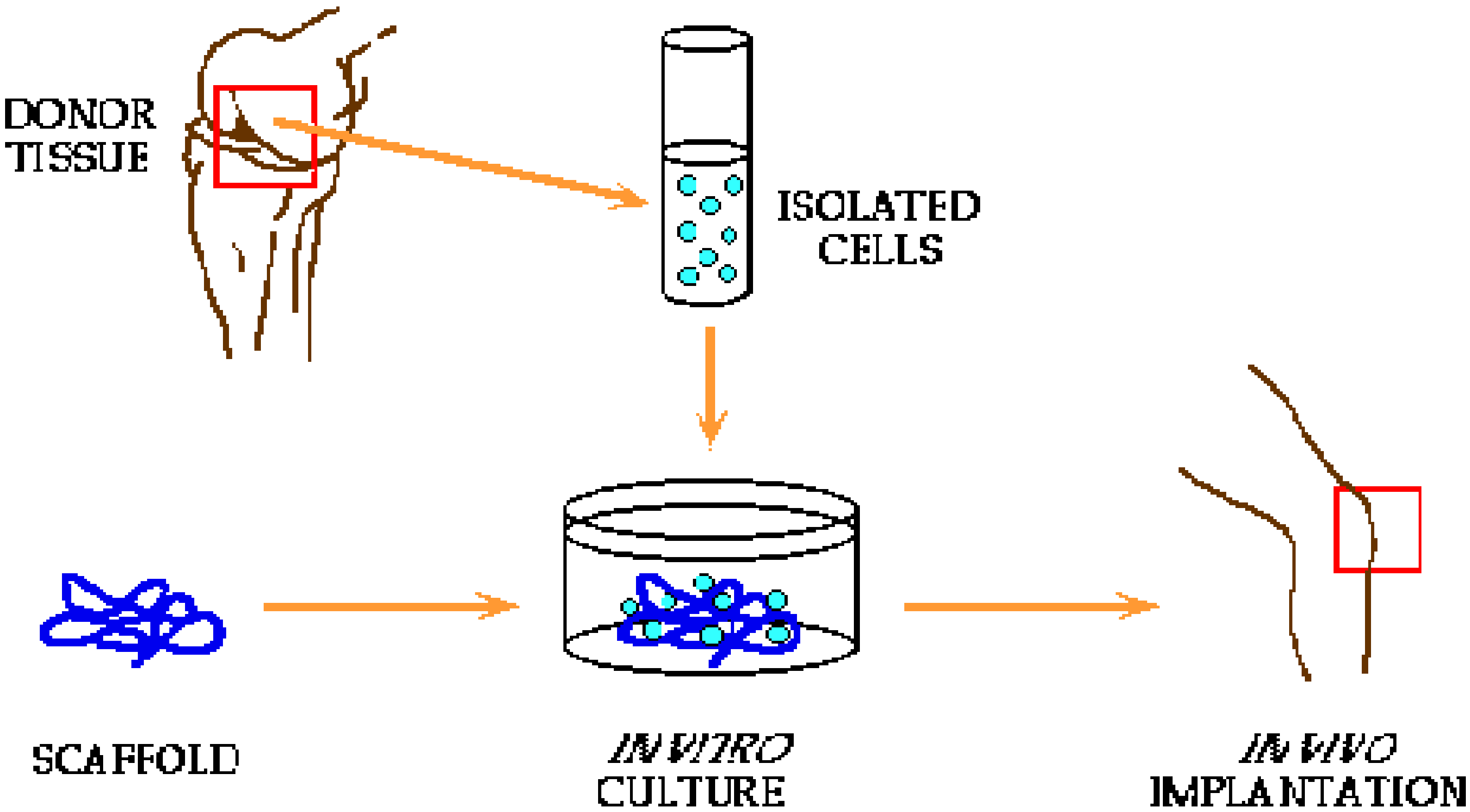
Controlling neuronal cell responses via LASER-fabricated 3D micro/nanostructured and patterned substrates

Anthi Ranella

Tissue Engineering



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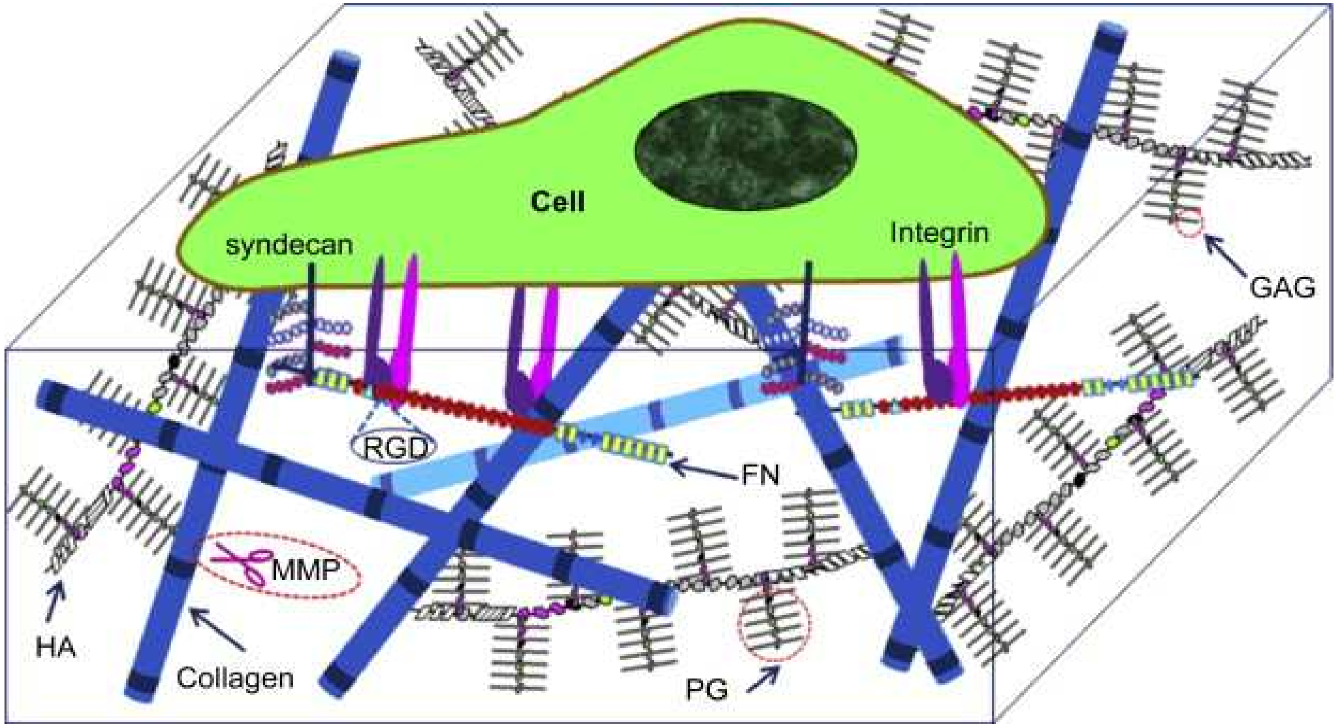
Model of complex 3D structure of extracellular matrix (ECM) and cell-ECM interactions.



Biomaterials, 2010, 31 (17), 4639

Science is built of facts
the way a house is built of bricks,
but an accumulation of facts is no more science
than a pile of bricks is a house

— Henri Poincaré

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How do cells interact with micro/nanostructures of well-defined sizes, at the molecular level?

Cell response dependence on tunable topography and defined chemistry

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Dr. Dina Terzaki

Laser based fabrication of biomimetic scaffolds

I. Ultrafast laser micro/nano structuring

Hierarchical micro/nano structuring

Dr Emmanuel Stratakis

II. 3-D nano-structuring using multi-photon polymerization

Sub-diffraction limit structuring

Dr. Maria Farsari

III. Single pulse UV laser irradiation of biopolymers

Micro porous foam structuring on natural biopolymers

Dr. Alexndros Selimis

Laser based fabrication of biomimetic scaffolds

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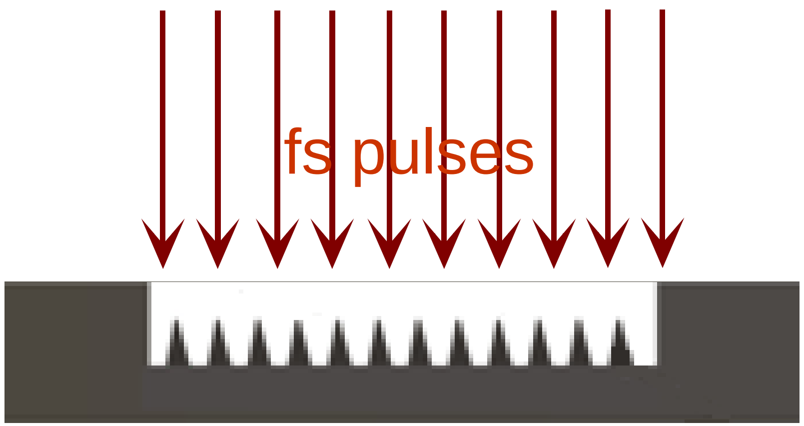
Micro porous foam structuring on natural biopolymers

Ultrafast laser micro/nano structuring

…a simple but effective method to fabricate silicon micro/nano structures over a large area with superior control of structure geometry and pattern regularity.

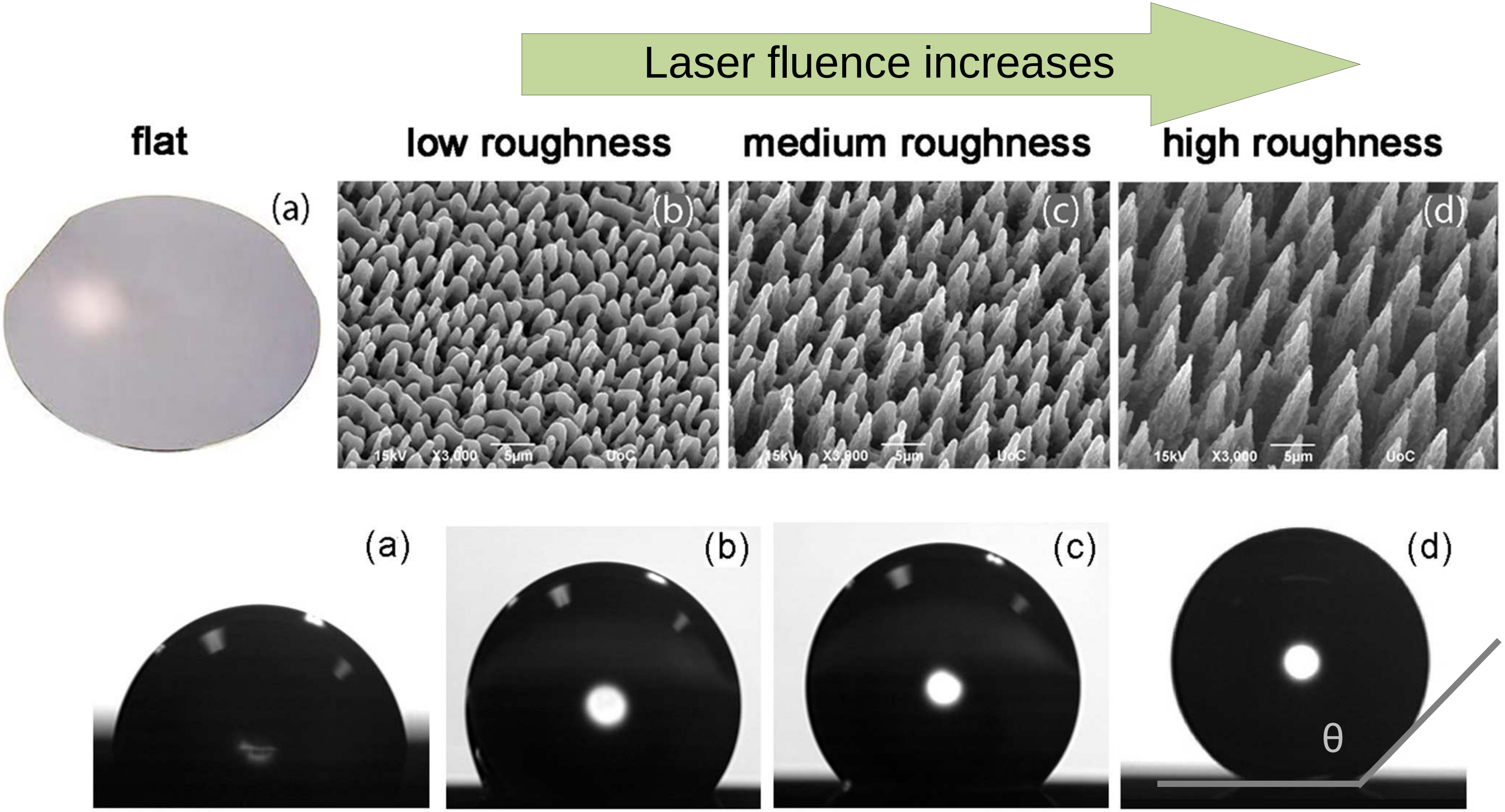
fs Laser irradiation of Si in a reactive gas atmosphere…

…can produce quasi-periodical structures exhibiting double scale roughness (Si spikes)



superior control of structure geometry and pattern regularity

Tailoring the Wettability of Solid Surfaces

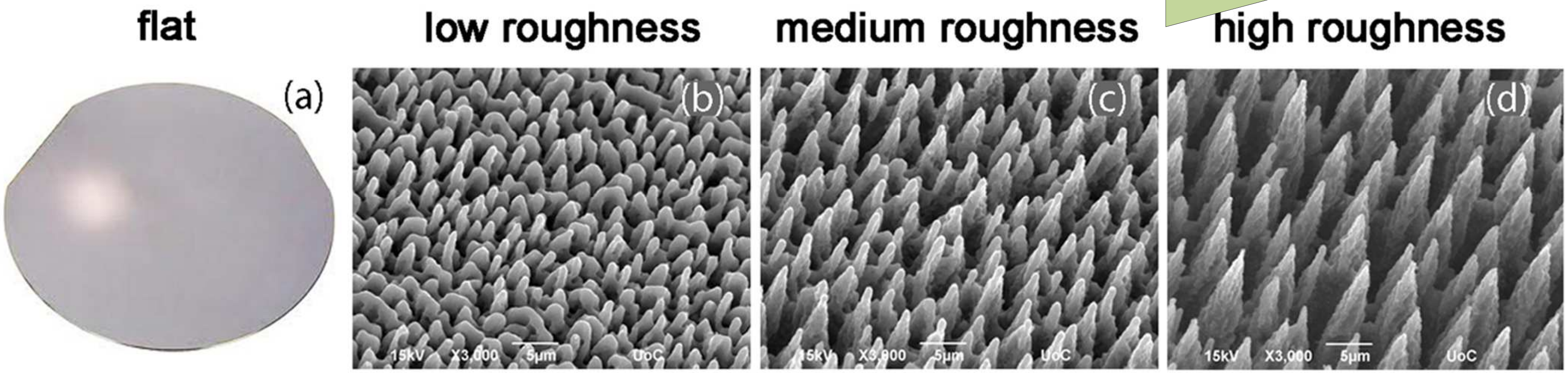


As microroughness ↑ ⟹ Surface Hydrophobicity ↑

V. Zorba et. al. Nanotechnology 17, 3234 (2006) & *Appl. Phys. A,* 93, 819 (2008)

Tailoring the Morphology of Solid Surfaces

Laser fluence increases →



Type of Roughness	Density D+STDEV (*10⁶/cm²)	Height, a ± STDEV (μm)	Base diameter, b ± STDEV (μm)	Aspect Ratio, A ± STDEV	Interspike Distance, c ± STDEV (μm)	Roughness Ratio, r ± STDEV
Low	9.75±1.54	1.26±0.28	1.87±0.60	1.41±0.27	1.73±0.28	1.74±0.23
Medium	5.01±0.19	3.76±0.42	2.17±0.25	3.51±0.52	2.06±0.53	3.65±0.49
High	2.50±0.26	8.63±1.17	4.78±1.03	3.73±0.80	4.48±0.96	3.83±0.76

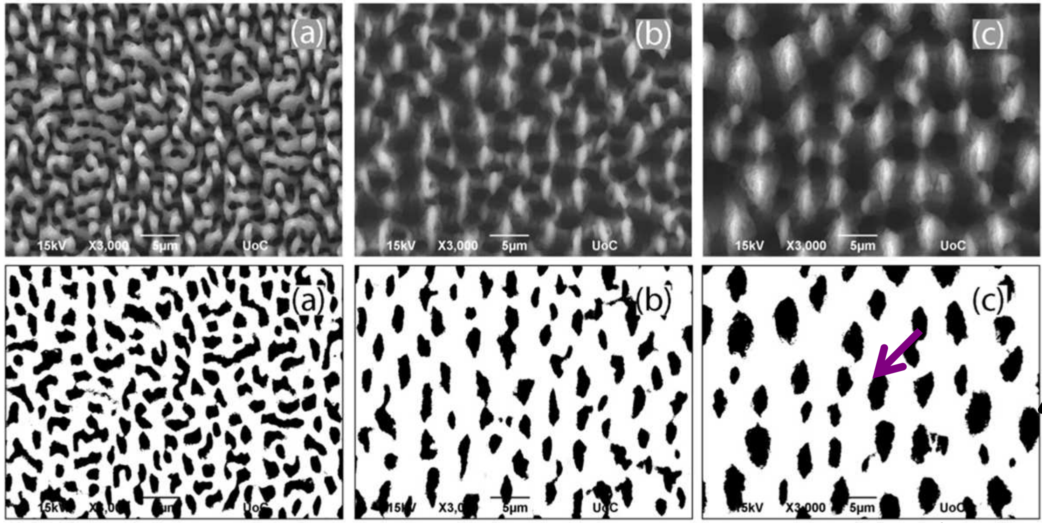
As roughness ↑ ⇒
- **Spikes' height ↑**
- **Interspike distance ↑**
- **Spike density ↓**

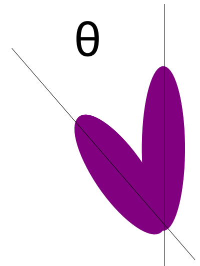
Ch. Simitzi, et al. J Tissue Eng Regen Med (2013)

Spike preferred orientation

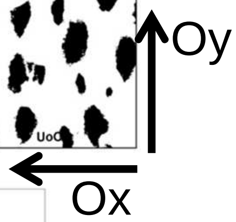
Top SEM views

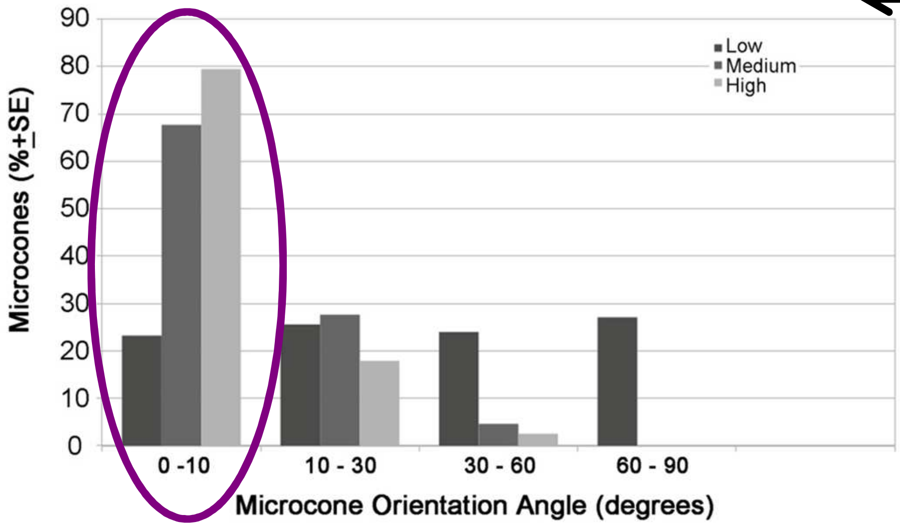
Thresholded & binarized images





With $0 < \theta < 90^{o}$





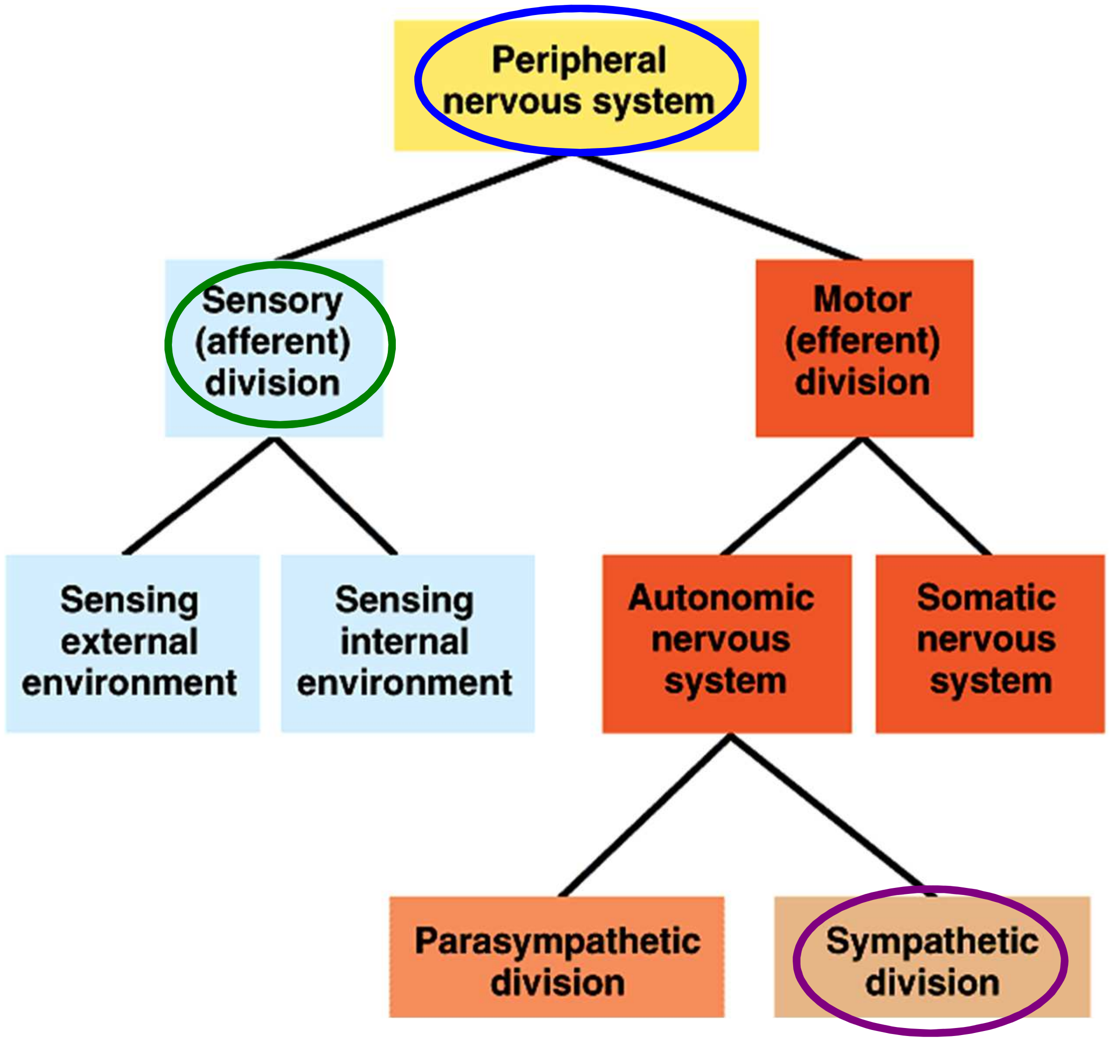
Whereas at the lower laser fluences the spikes don't seem to exhibit a preferred orientation, **as the laser fluence increases, the spikes present a striking parallell aligned orientation**

Microconical silicon substrates as cell culture platforms

Therefore, the suggested topography could be described as **semiperiodical discontinuous** (arrays of oriented microcones) comprising an **anisotropic feature** (elliptical cross-section).

Microconical silicon substrates as cell culture platforms

The simplicity of the irradiation process offers the possibility of patterning areas with different degrees of roughness on the same culture substrate.



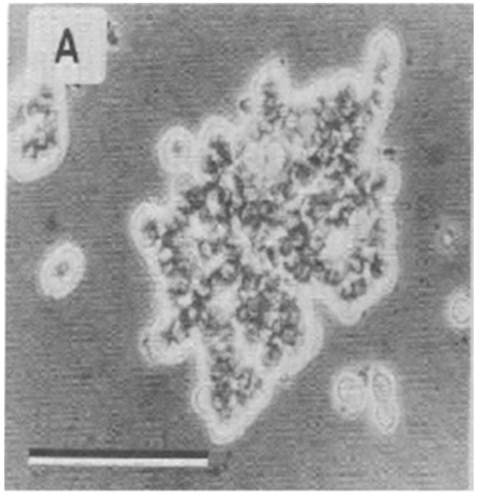
Neuron-like cell line

- PC12 cells

Primary cultures

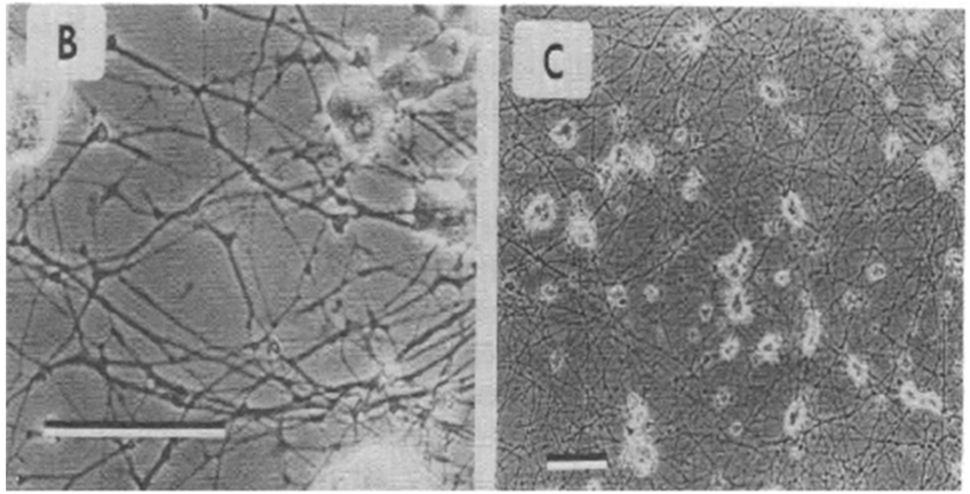
- Schwann cells
- Sympathetic neurons (Superior cervical ganglia)
- Sensory neurons (Dorsal Root Ganglia)

PC12 cells (Pheochromocytoma cells)



Scalebar:100μm

Nerve Growth Factor

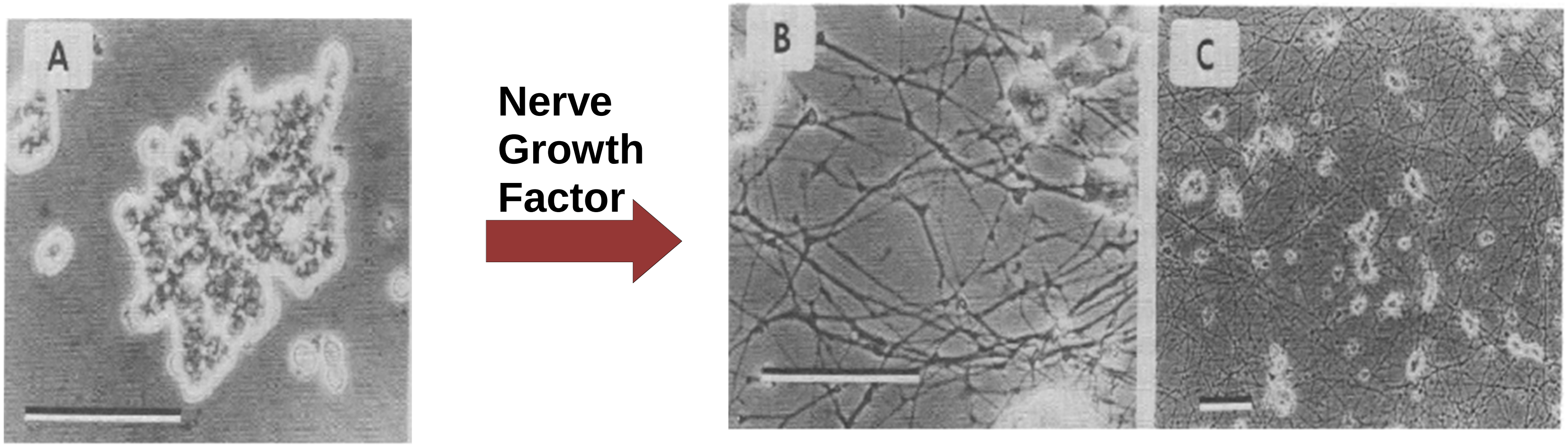


14 DIV **22 DIV**

“The cells tended to form clumps composed of 5-20 cells”

Greene & Tischler 1976

PC12 cells
(Pheochromocytoma cells)



14 DIV 22 DIV

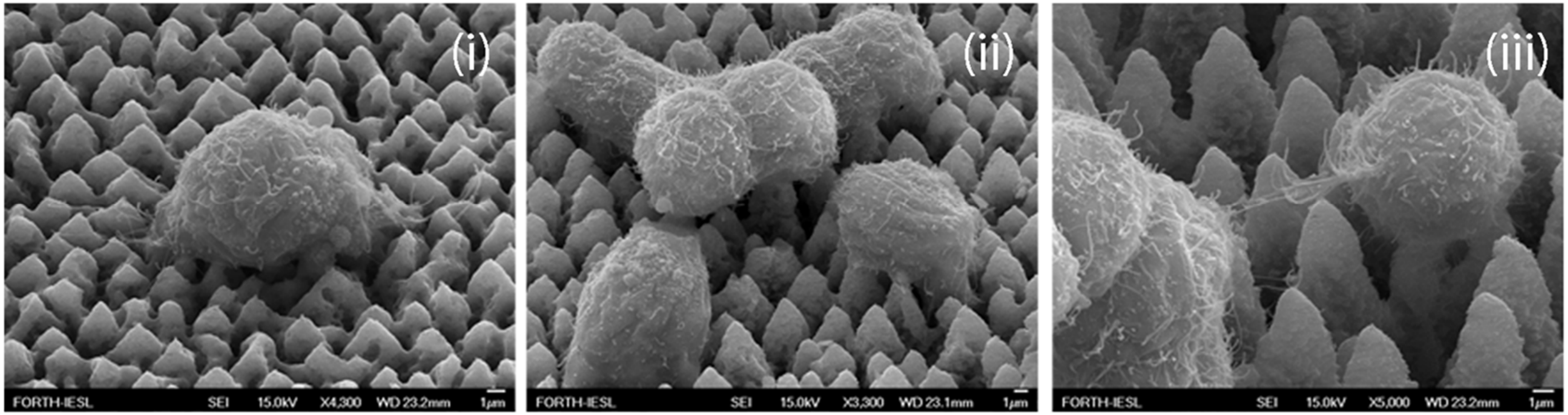
Scalebar:100μm

In the presence of Nerve Growth Factor (NGF)
they obtain the phenotype of **sympathetic neurons**
(they develop processes, have varicosities and become electrically excitable)

Greene & Tischler 1976

PC12 cells are a useful model for the study of neuronal differentiation at cellular & molecular level

Effect of surface roughness on PC12 cell growth

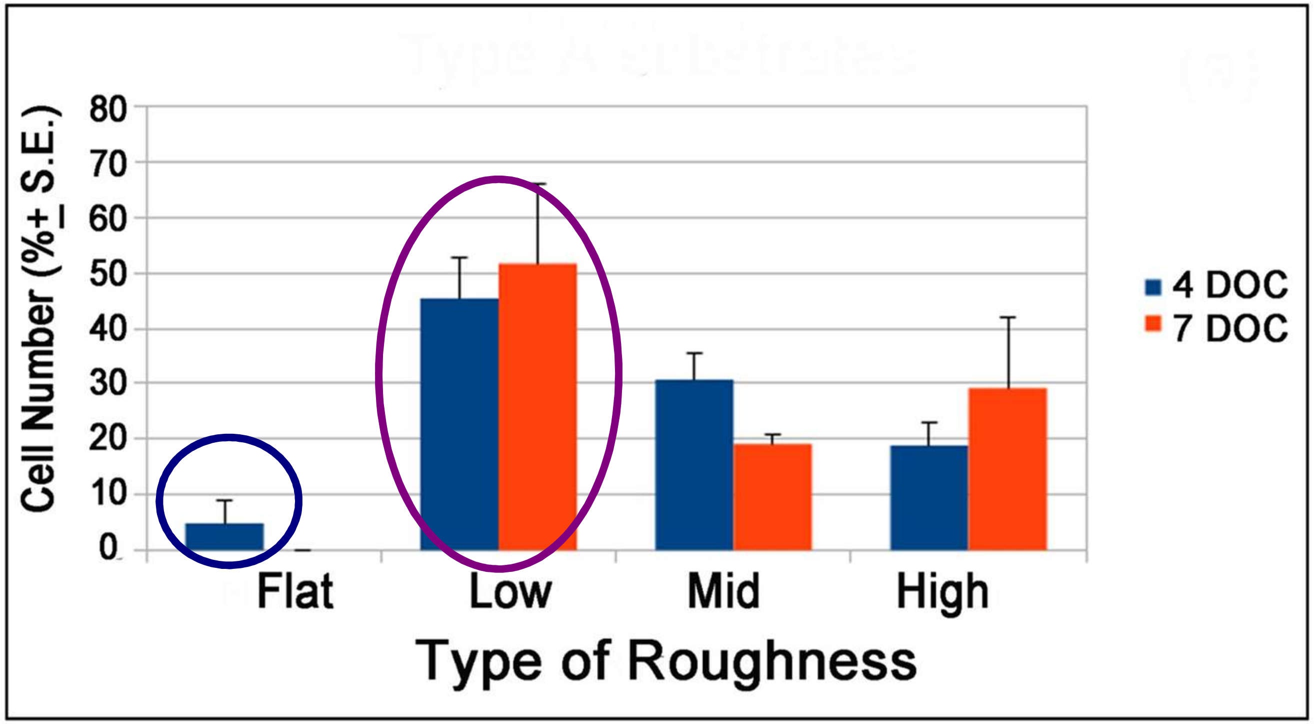


Low roughness **Medium roughness** **High roughness**

PC12 cells were grown on all three roughness types, while sharing the same morphological characteristics, including the relatively **small and rounded shape cluster** formation

Ch. Simitzi, et al. J Tissue Eng Regen Med (2013)

Effect of surface roughness on PC12 cell growth

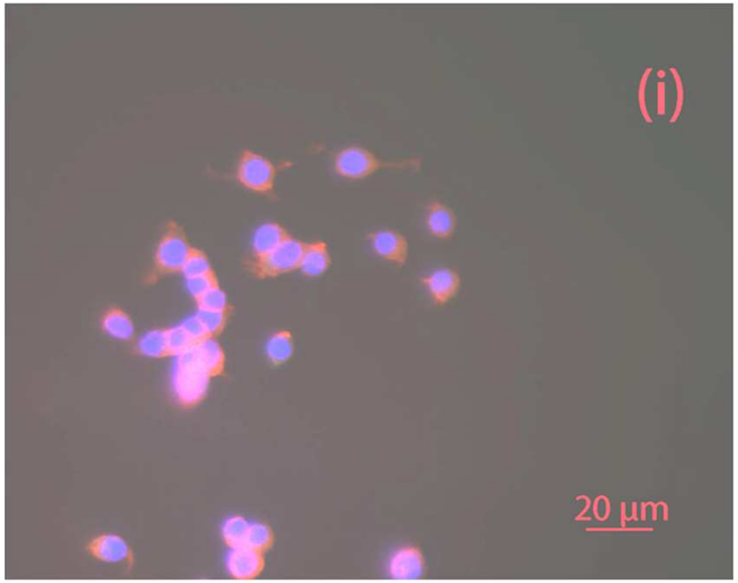


Among the different non coated roughness substrates, PC12 cells seemed to prefer the **low** roughness structures

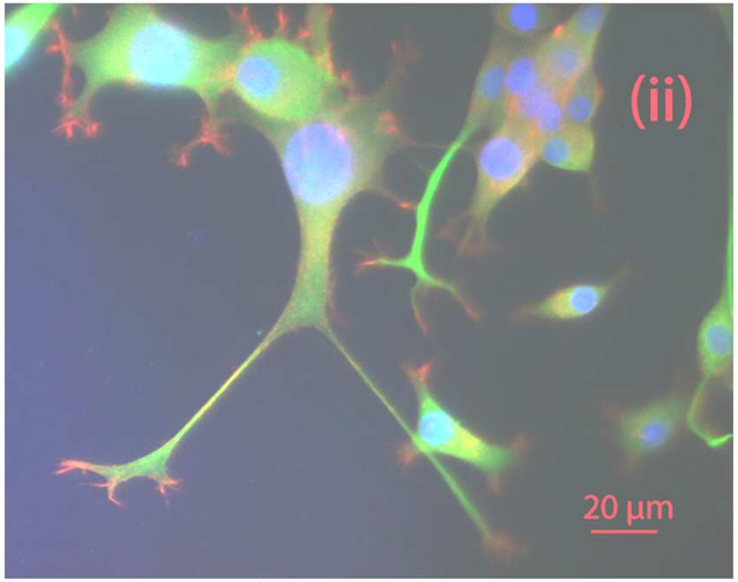
MCs surfaces were largely preferred as compared to flat ones
(2- to 8-fold higher proliferation while flat surfaces could not support cell growth after 7 days of culture

Effect of surface roughness on PC12 cell growth in the presence of NGF

DAPI: nuclei
Actin
Tubulin



Without NGF



With NGF

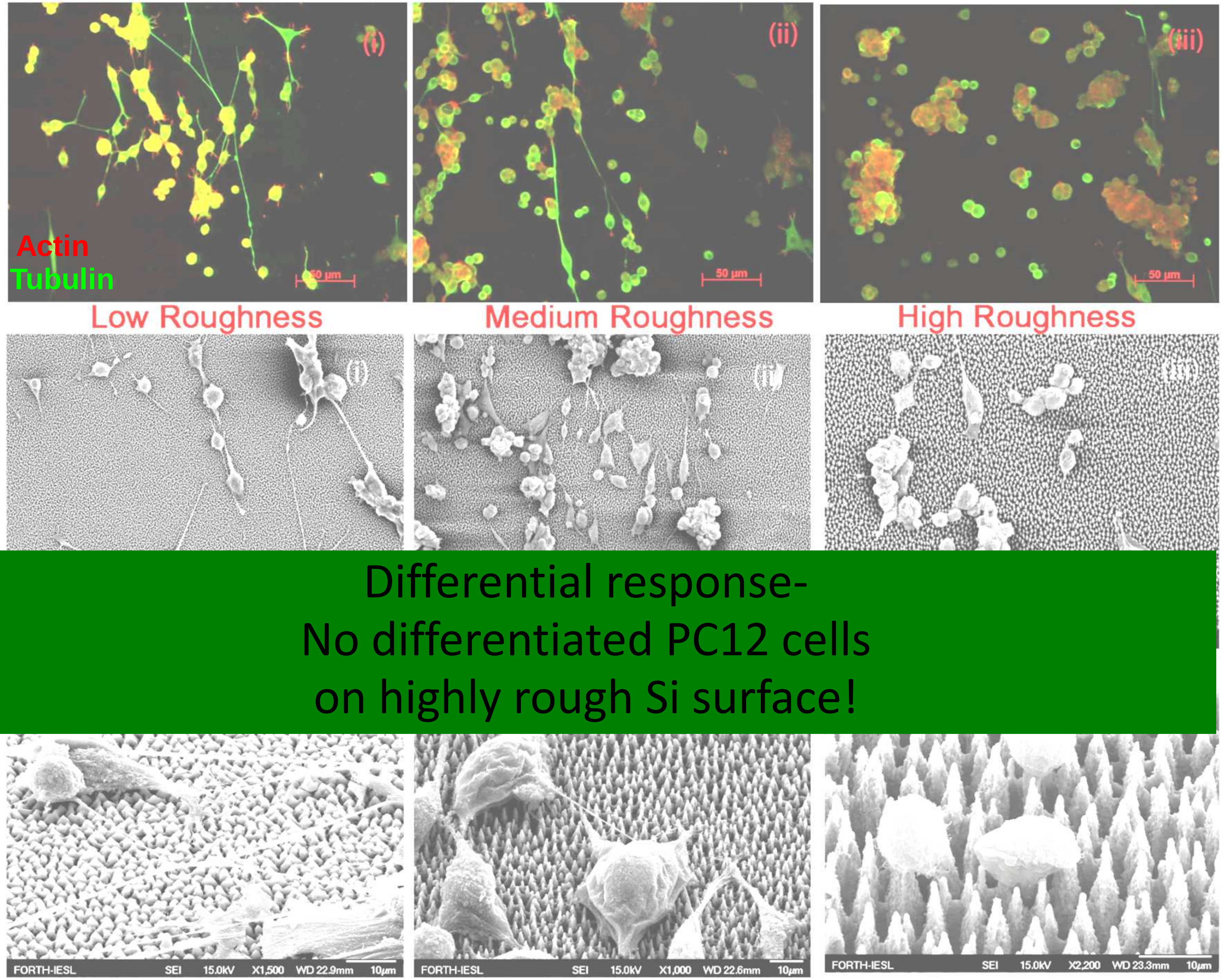
The PC12 cells growing on the low and mid roughness MCs could differentiate towards the neuronal cell lineage, showing increased, flattened cellular body, sprouting neuritic processes

Ch. Simitzi, et al. J Tissue Eng Regen Med (2013)

Effect of surface roughness on PC12 cell differentiation

Fluorescence microscopy images

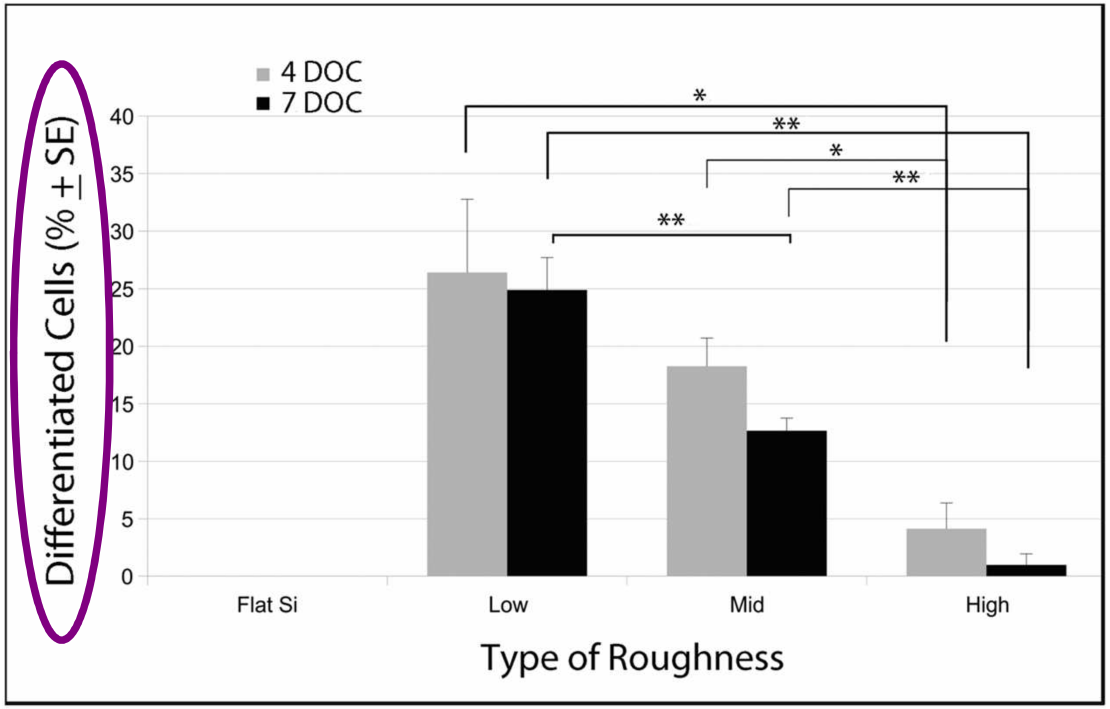
SEM images



Differential response-
No differentiated PC12 cells
on highly rough Si surface!

Effect of surface roughness on PC12 cell growth in the presence of NGF

Differentiated cells (%):
Cells with Neurites/total cells



(I)

$L_{neurite}$: The mean length of the longest neurite per cell

Roughness Type	4DOC $L_{neurite}$ (μm)	7DOC $L_{neurite}$ (μm)
Low	66.7± 9.6	79.0± 7.4
Mid	41.1± 3.7	82.7 ±12.3
High	28.0± 4.2	30.1± 5.5

(II)

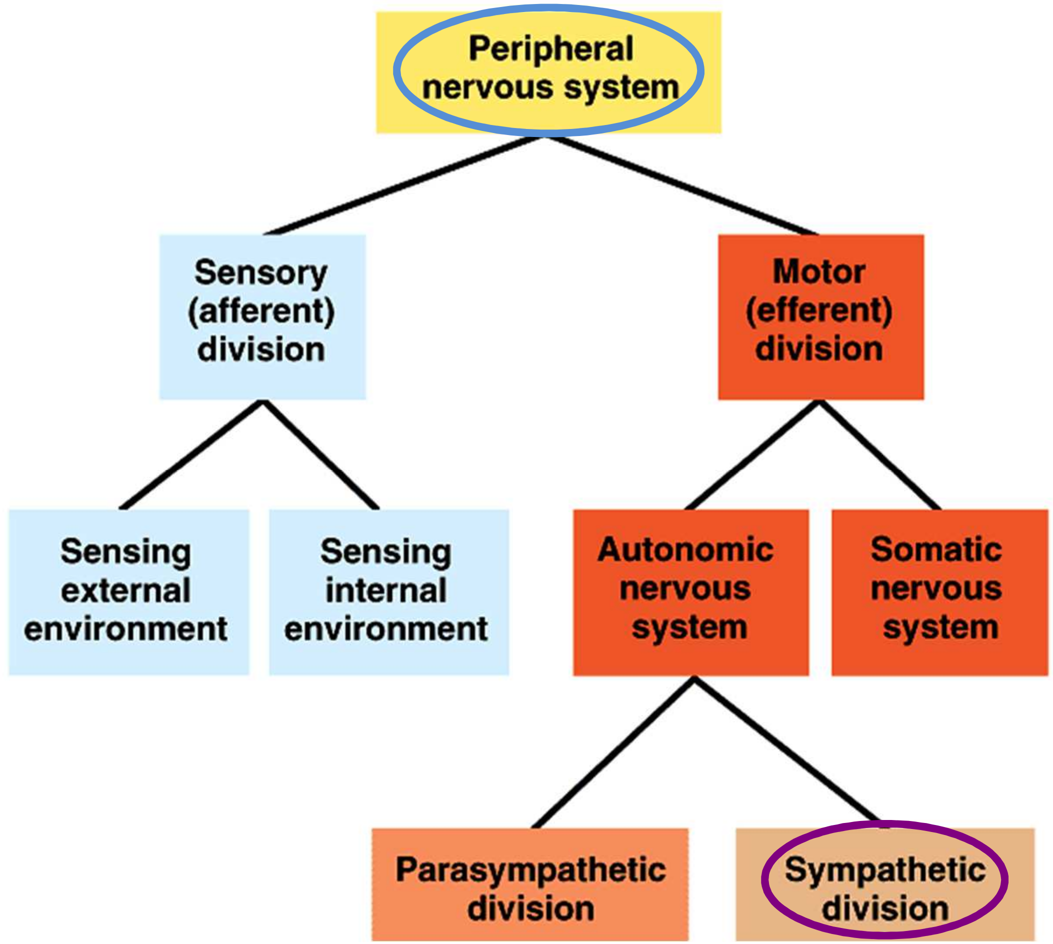
Ch. Simitzi, et al. J Tissue Eng Regen Med (2013)

PC12 cell line

a correlation between the geometrical characteristics of the topographical features of the surfaces and the (respective) cell responses

How do primary cells of the nervous system respond to the underlying surface topography?

In vitro experiments with cells



Neuron-like cell line

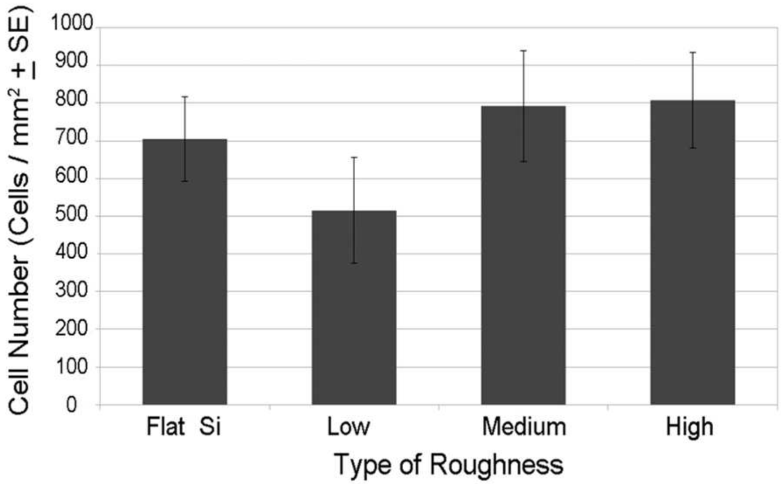
- PC12 cells

Primary cultures

- **Schwann cells**
- **Sympathetic neurons (Superior cervical ganglia)**
- Sensory neurons (Dorsal Root Ganglia)

Primary cells on μ-patterned Si substrates

Schwann cells



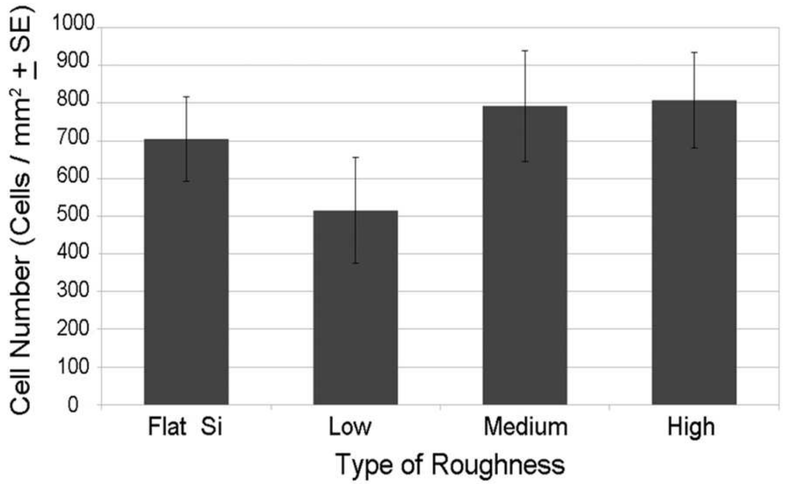
All three micro-patterned Si substrates could equally well support the growth of Schwann cells

Culture medium: DMEM + 1% FBS
Culture time: 7 DOC

C Simitzi, P Efstathopoulos, et al. 2015 Biomaterials 67:115-128

Primary cells on μ-patterned Si substrates

Schwann cells



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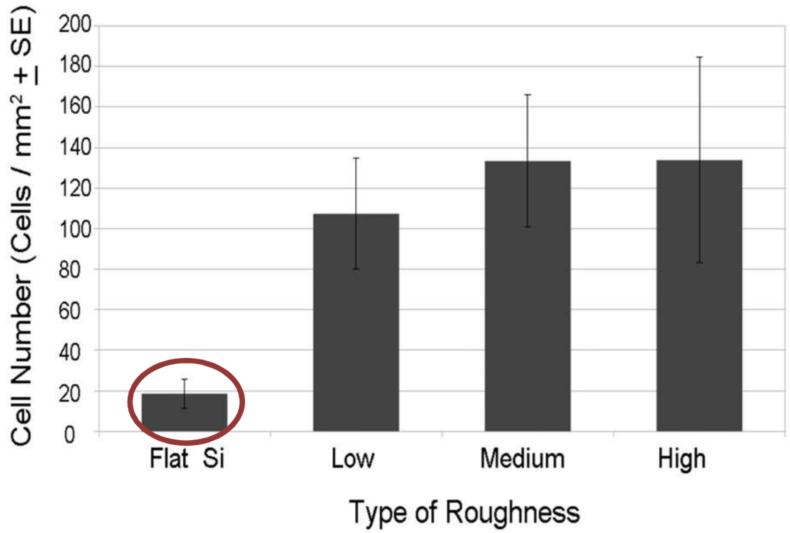
Sympathetic neurons

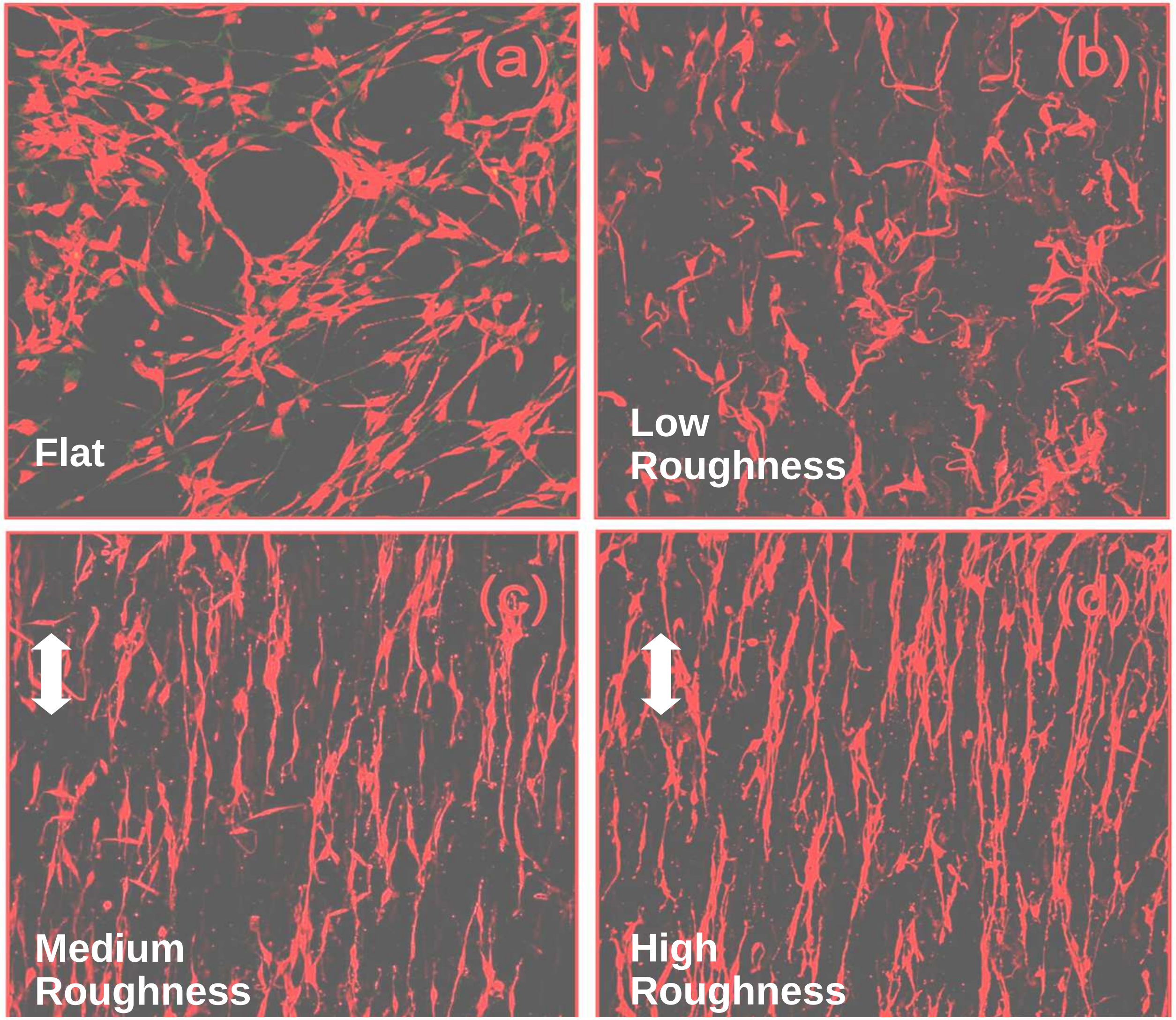


Although very few neurons could grow on the flat Si substrates, all micropatterned Si substrates did support extended neuronal outgrowth.

Coating: Collagen solution
Culture medium: RPMI + 1% FBS+ 100 ng/ml NGF
Culture time: 7 DOC

C Simitzi, P Efstathopoulos, et al. 2015 Biomaterials 67:115-128

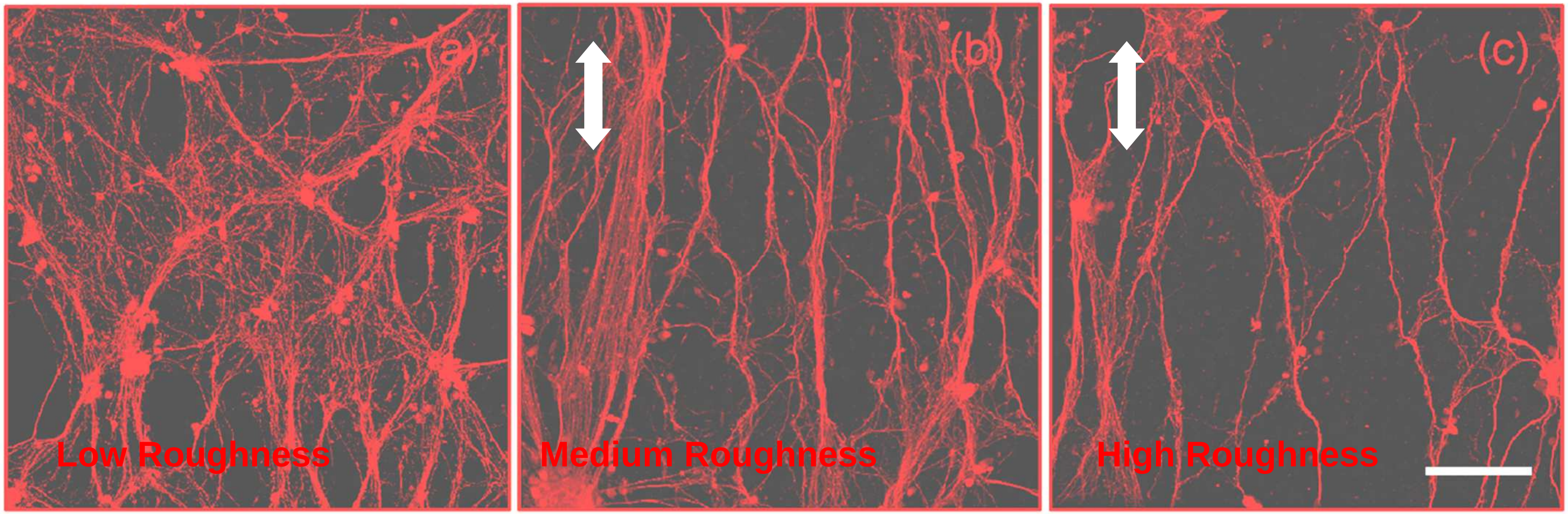
Schwann cells on μ-patterned Si substrates



S100

Remarkably, there is a trend for **preferred outgrowth orientation** on mid and high roughness substrates

Sympathetic neurons on μ-patterned Si substrates



Isotropic outgrowth	Parallel alignment of neurons	Parallel alignment of neurons

Topography-dependent axonal outgrowth pattern:

Axons on the low roughness substrates were shown to grow randomly, whereas axons on medium and high roughness substrates followed a parallel alignment growth pattern.

C Simitzi, P Efstathopoulos 2015 Biomaterials 67:115-128

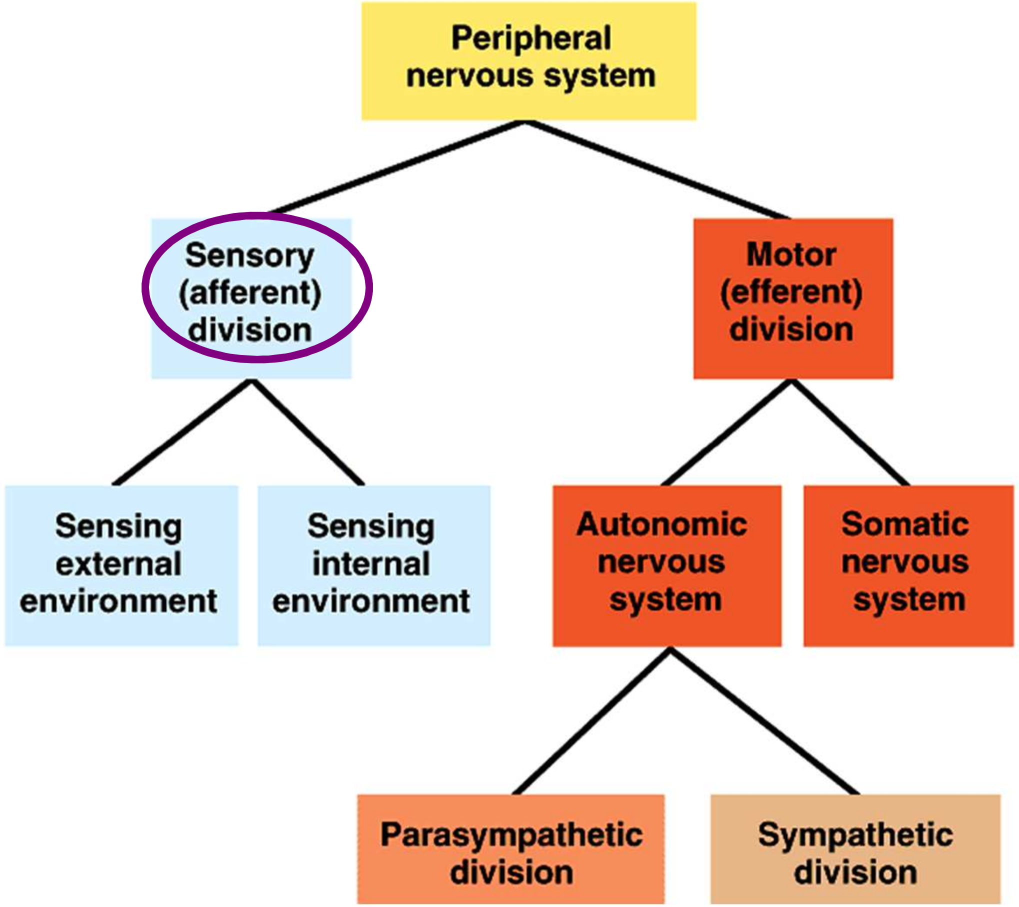
Topographic guidance of neural cell outgrowth

- *The importance of surface roughness over nerve cell outgrowth and network formation is emphasized*

- *Axonal outgrowth pattern was dependent on the underlying topography*

- *Preferred Schwann cell outgrowth orientation towards the substrates with increasing roughness.*

In vitro experiments with cells



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Primary cultures

- Schwann cells
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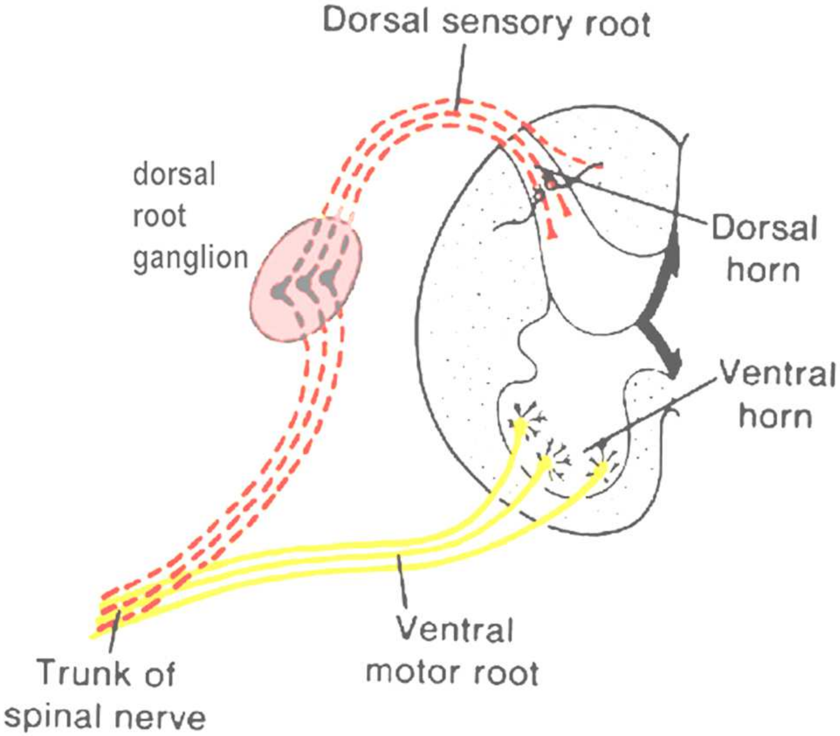
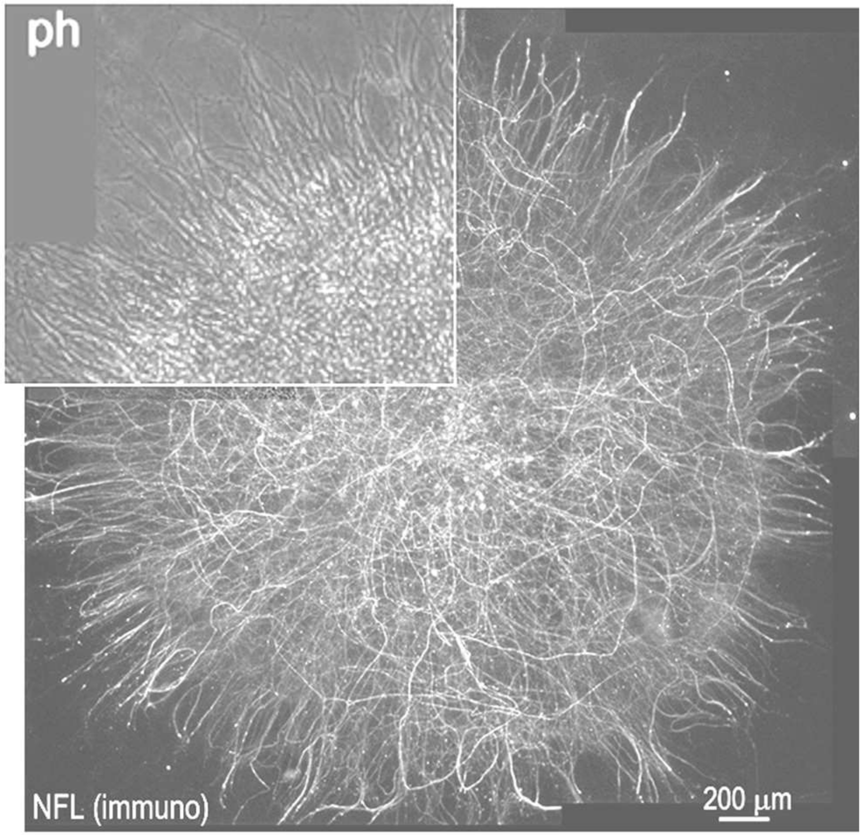
- **Sensory neurons (Dorsal Root Ganglia)**

DRG whole explants





A phase image (ph) and immunofluorescence image of neurofilament protein (NFL, axonal marker)

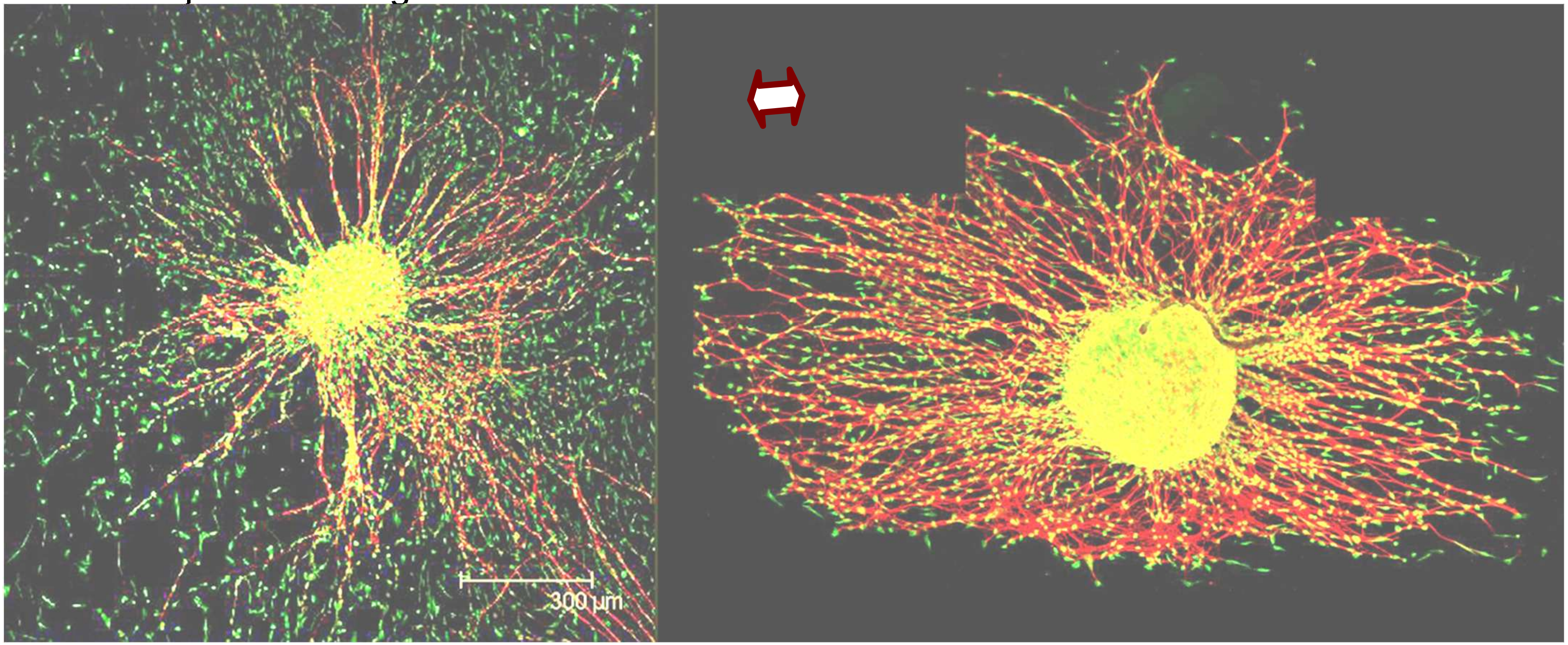
Dorsal root ganglia (DRG) are **collections of sensory nerve bodies, their axons and Schwann cells** located posterolateral to the spinal cord

These can be isolated from embryonic mice and grown in culture, allowing one to follow the process of axonal myelination.

Whole dorsal root ganglion explants

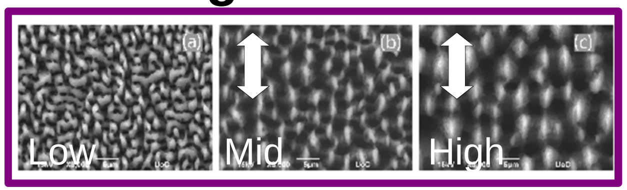
Low Roughness
Isotropic Cell Outgrowth

High Roughness
Anisotropic Cell Outgrowth

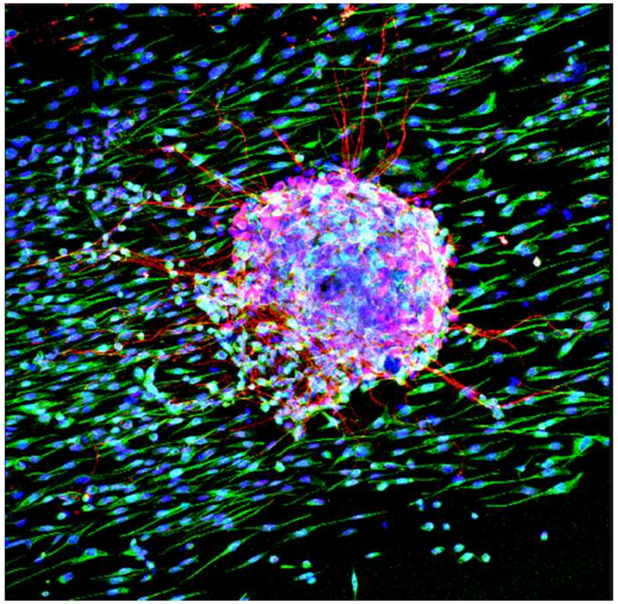


S100: Schwann Cells NF: Neurons

The trend for preferred orientation of cell migration and axonal outgrowth is enhanced as the surface roughness increases

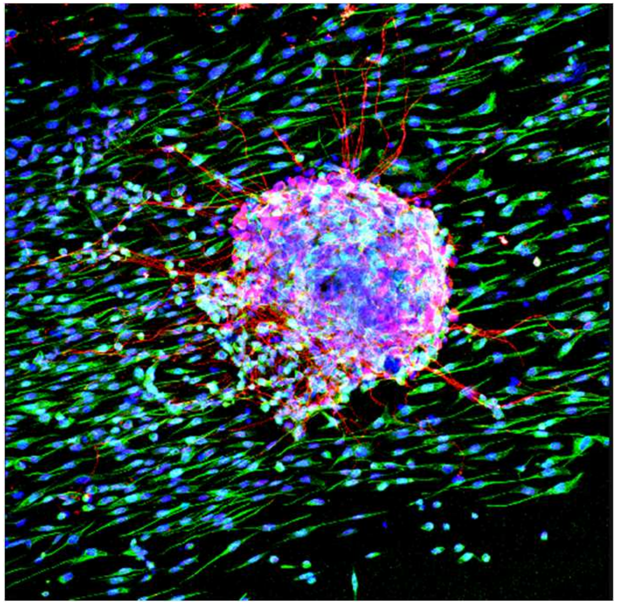


Spatial relationships between axons & non-neuronal cells



S100: Schwann cells
Neurofilament: Axons
To-Pro: Cell nuclei

Spatial relationships between axons & non-neuronal cells



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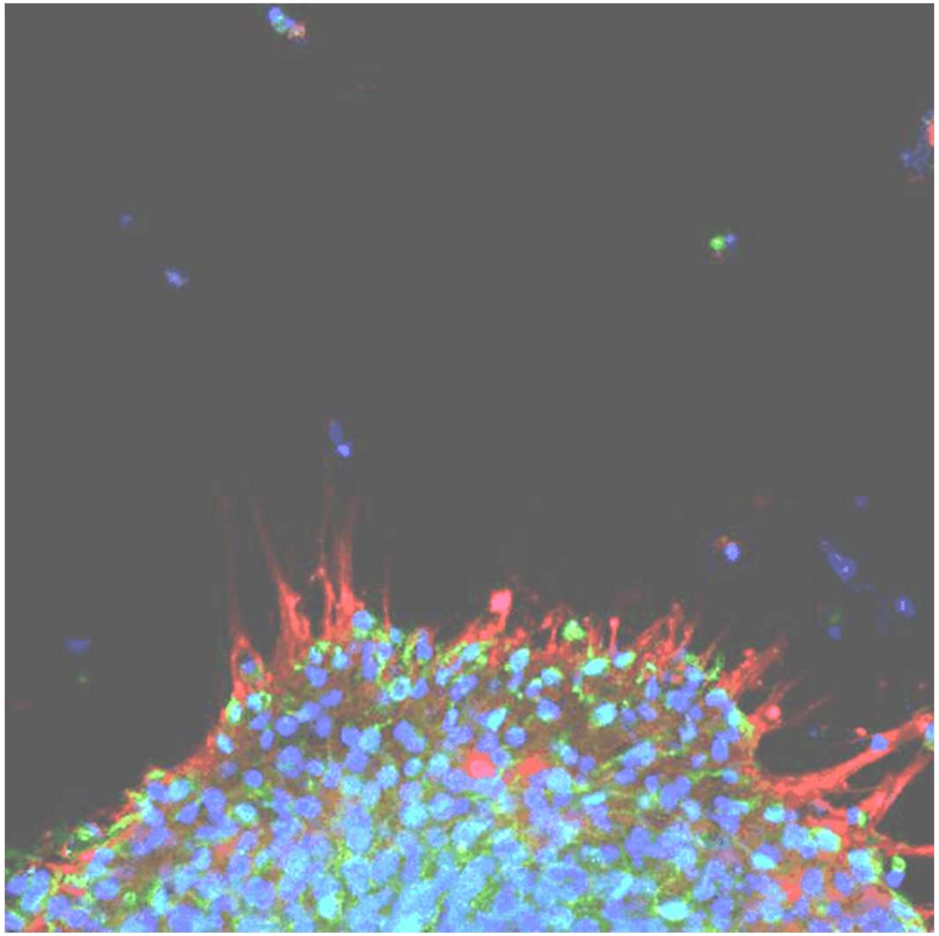

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Schwann cell migration and axonal outgrowth on micropatterned Si surfaces

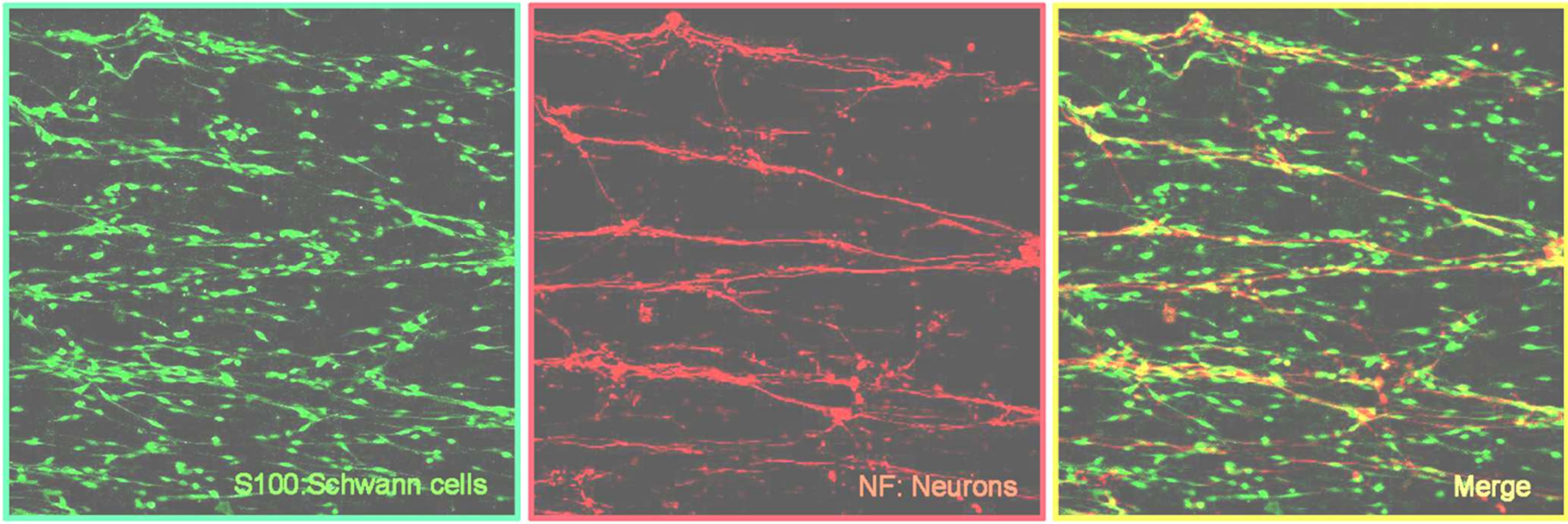
- The plasticity of the Schwann cells and their processes allowed them to create a “carpet”
- This glial cell “carpet” served as a substrate for the outgrown neurites

↓

Schwann cells were guided by the underlying topographical features of the micropatterned silicon surface

Neurons were, in turn, outgrown on top of them

Co-culture of Schwann cells & SCGs neurons on μ-patterned Si substrates



Neurite Outgrowth Is Directed by Schwann Cell Alignment in the Absence of Other Guidance Cues!

Conclusion

-PC12 cell line

The cell response (**outgrowth** & **differentiation**) was influenced by the substrate topography

-Primary ganglion cell cultures

-The micropatterned substrates can support ganglion explant nerve cell outgrowth, without the need for coating

-The micropatterned substrates can support outgrowth and network formation of dissociated ganglion neurons.

-There is a trend for **preferred outgrowth orientation** of Schwann cell on mid and high roughness substrates

⇩

a correlation between the geometrical characteristics of the topographical features of the surfaces and the (respective) cell responses

Laser based fabrication of biomimetic scaffolds

I. Ultrafast laser surface's modification

Hierarchical micro/nano structuring

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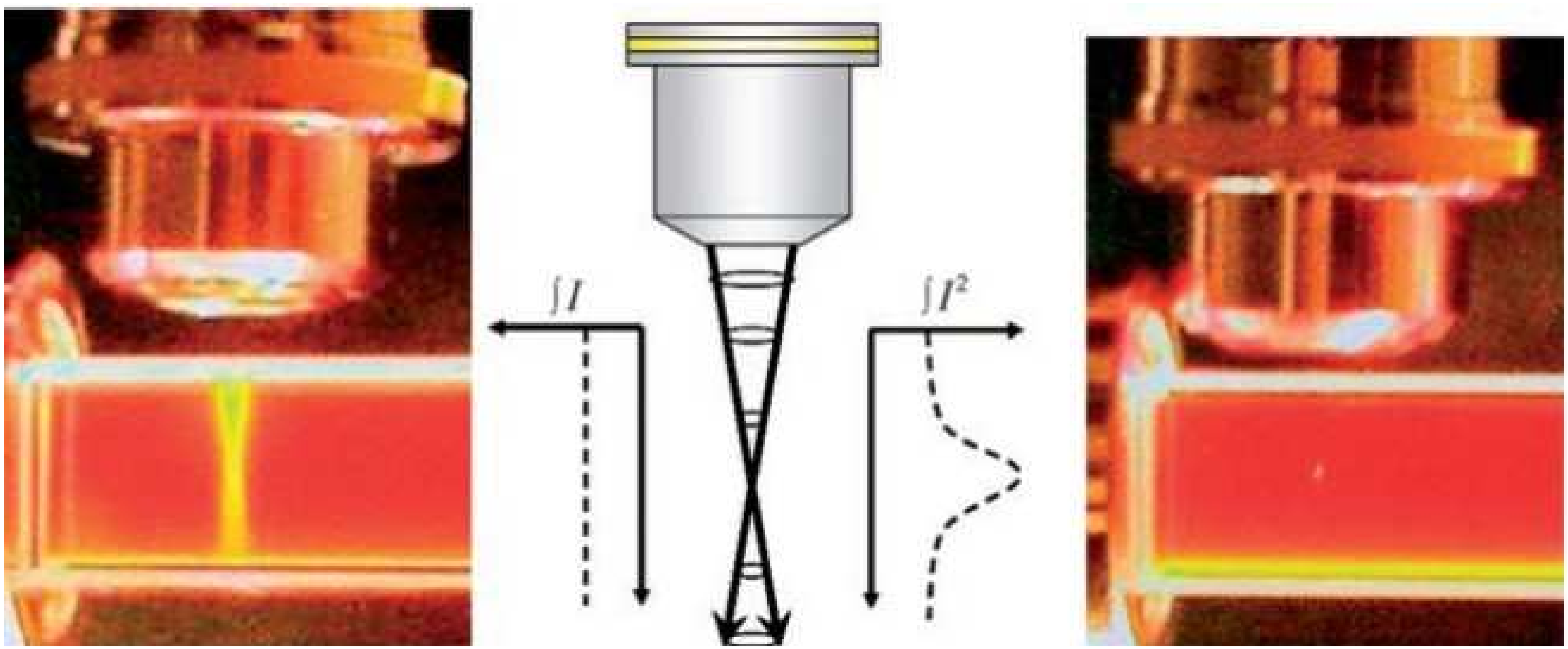
Micro porous foam structuring on natural biopolymers

3-D nano-structuring using multi-photon polymerization

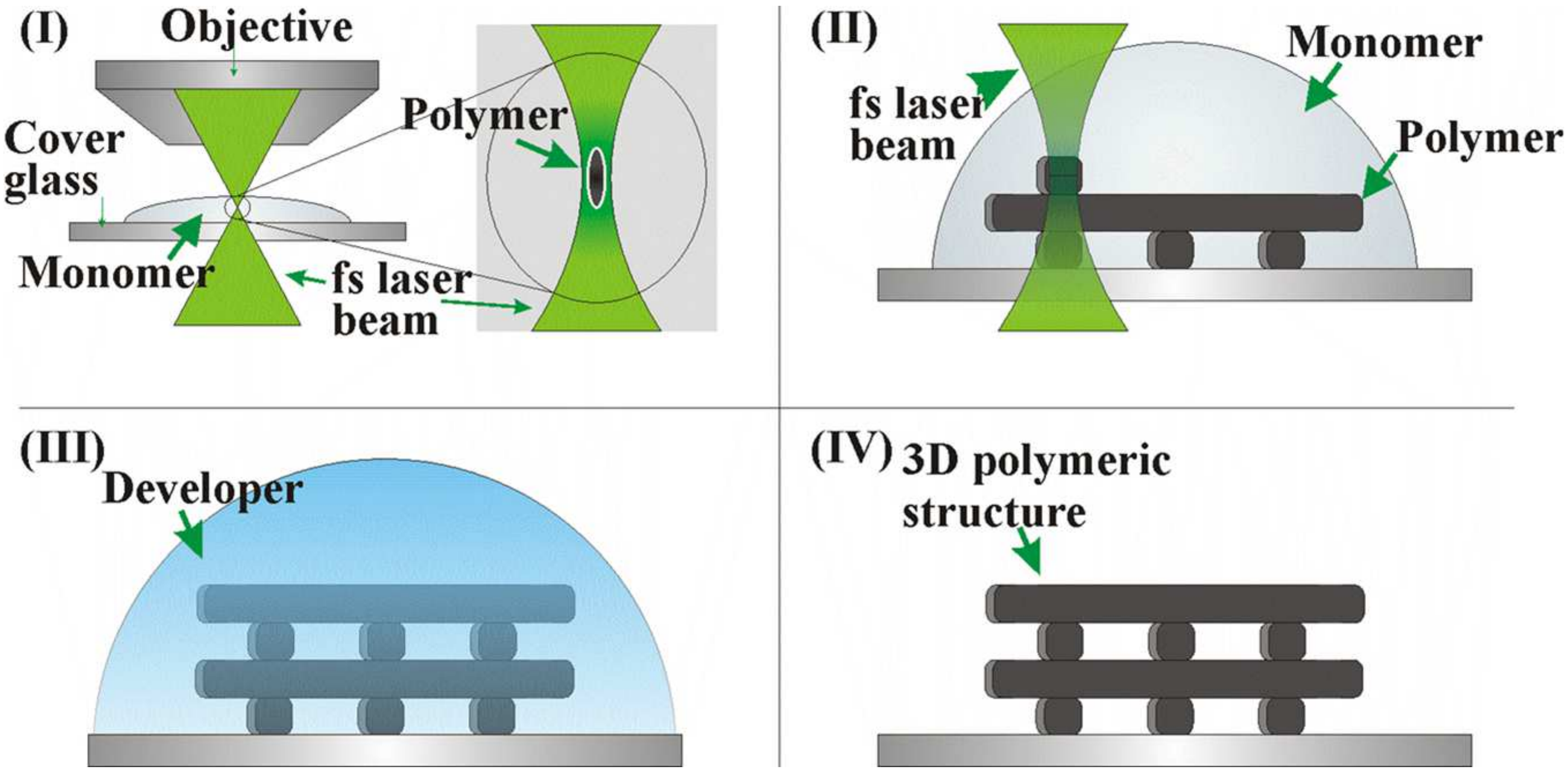
1-photon absorption

2-photon absorption



Picture: C.N. LaFatta et al., Angew. Chem. Int. Ed. 2007, 46, 6238 – 6258.

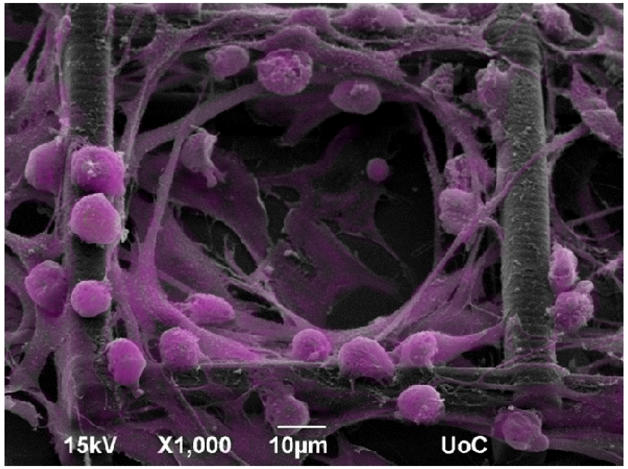
3-D nano-structuring using multi-photon polymerization

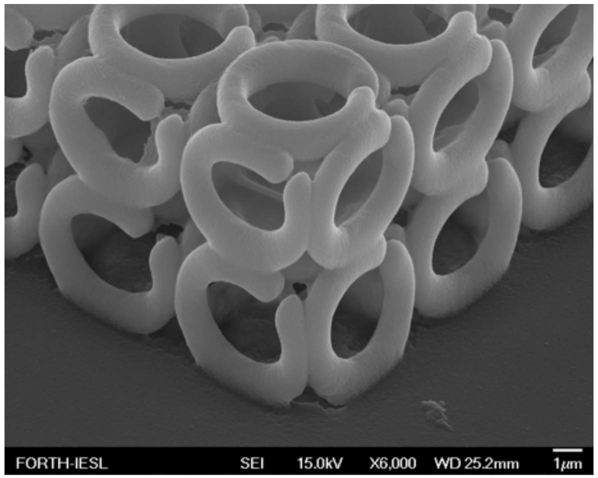


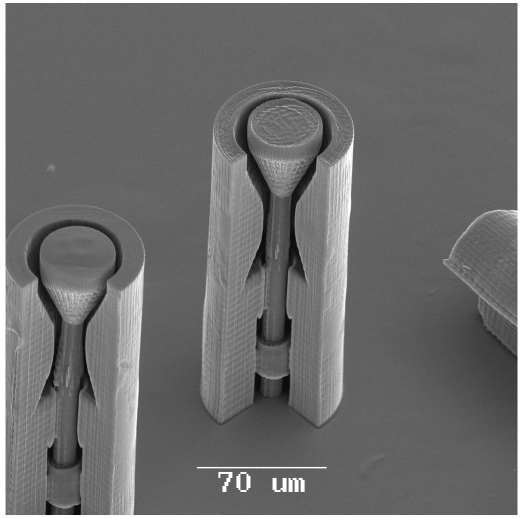
Laser source, Ti:Sapphire:
λ=800 nm, τ_{pulse}<20 fs, repetition rate = 75 MHz

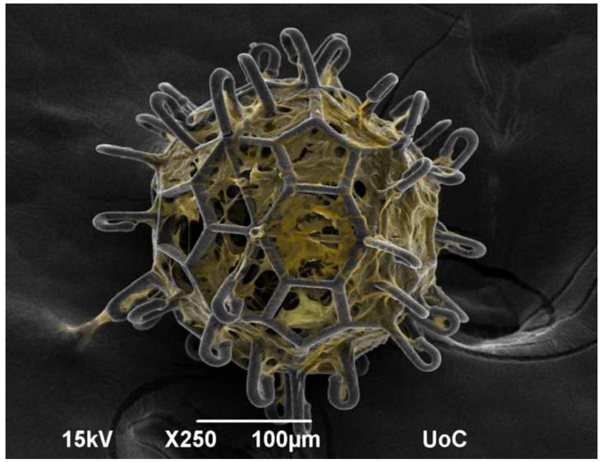
3-D nano-structuring using multi-photon polymerization

<u>Photopolymer</u>
Biocompatible, non toxic,
non-biodegradable,
Hybrid (organic/inorganic)



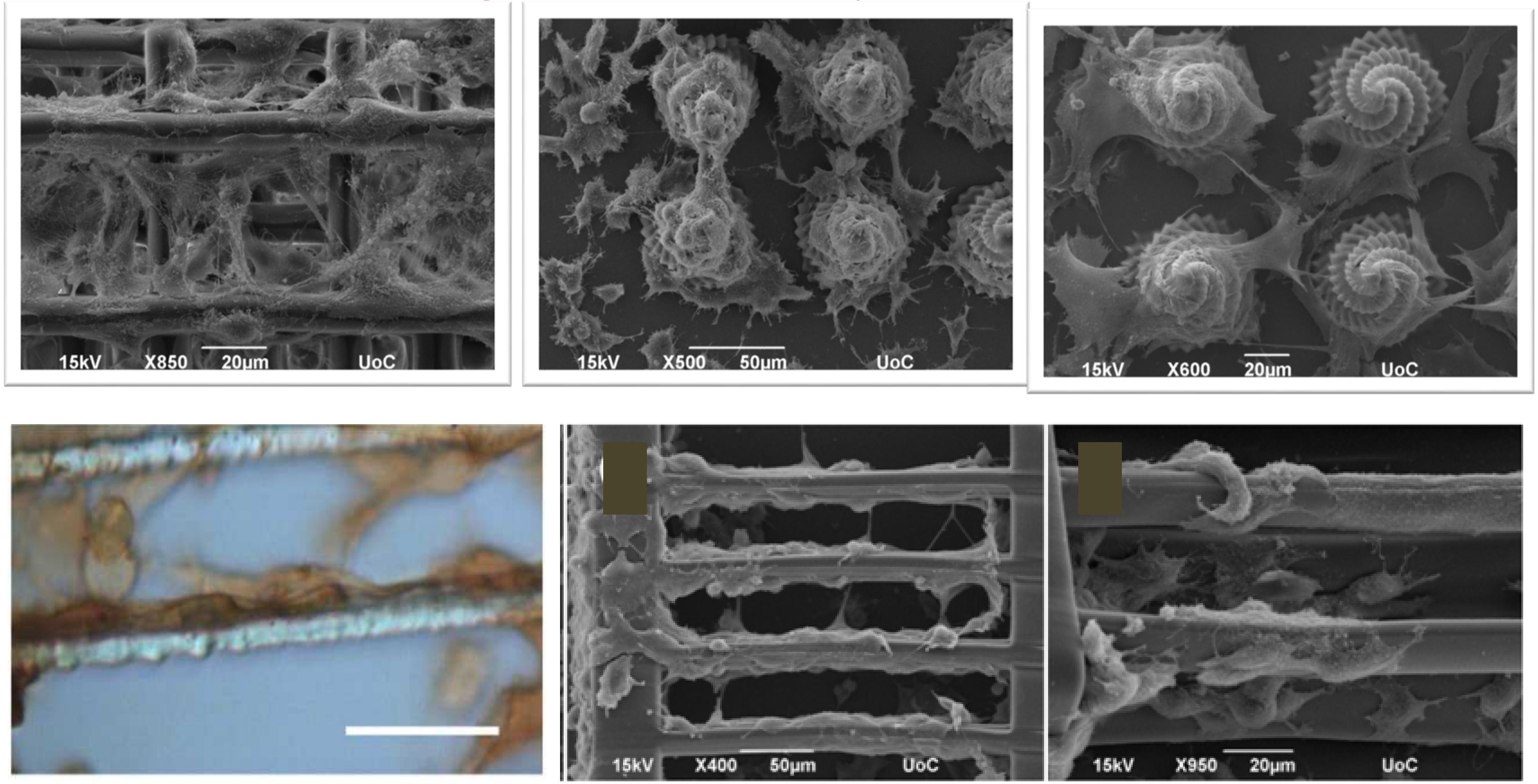






3-D nano-structuring using multi-photon polymerization

Polylactide-based biodegradable photopolymer



"Direct Laser Writing of 3D scaffolds for neural tissue engineering applications," V. Mellisinaki, *et al.*, *Biofabrication*, 2011.

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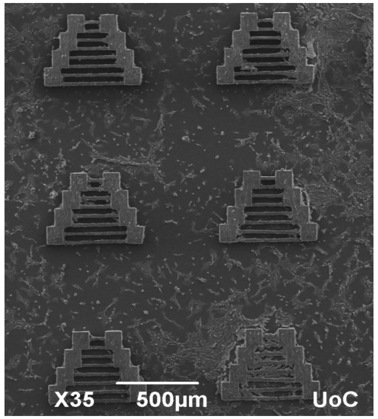
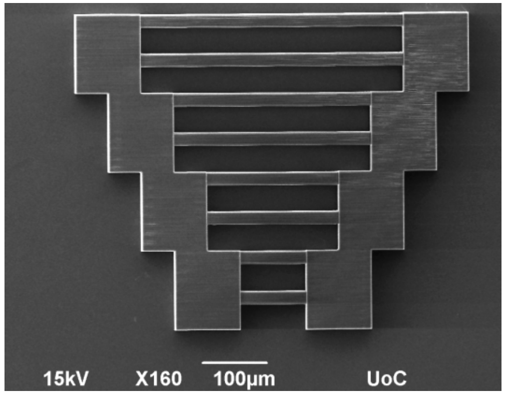
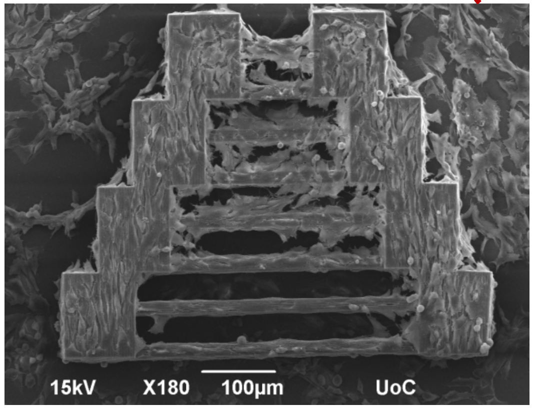
3-D nano-structuring using multi-photon polymerization

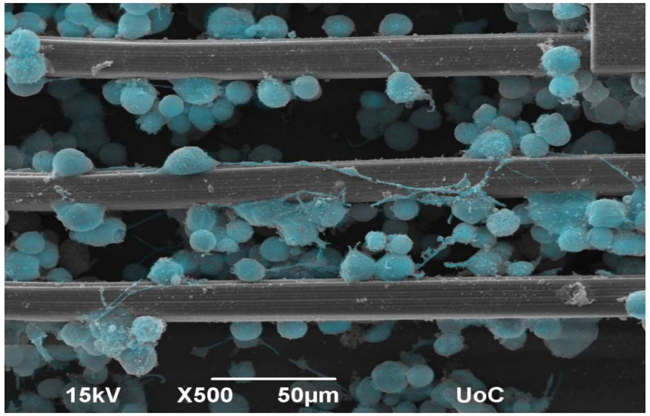


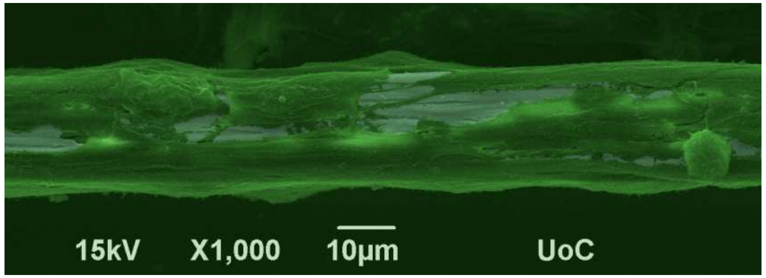


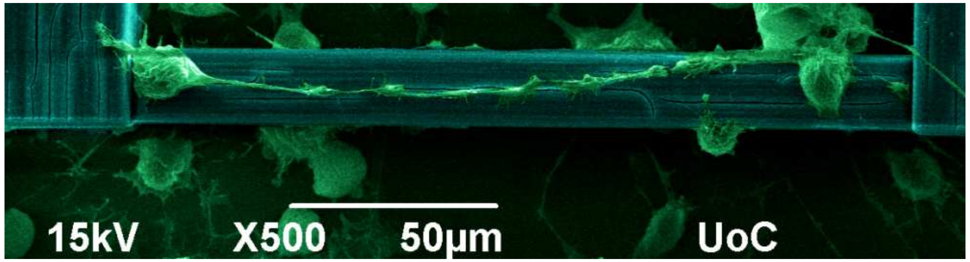
Schwann cells (SW10)



NEURO2A







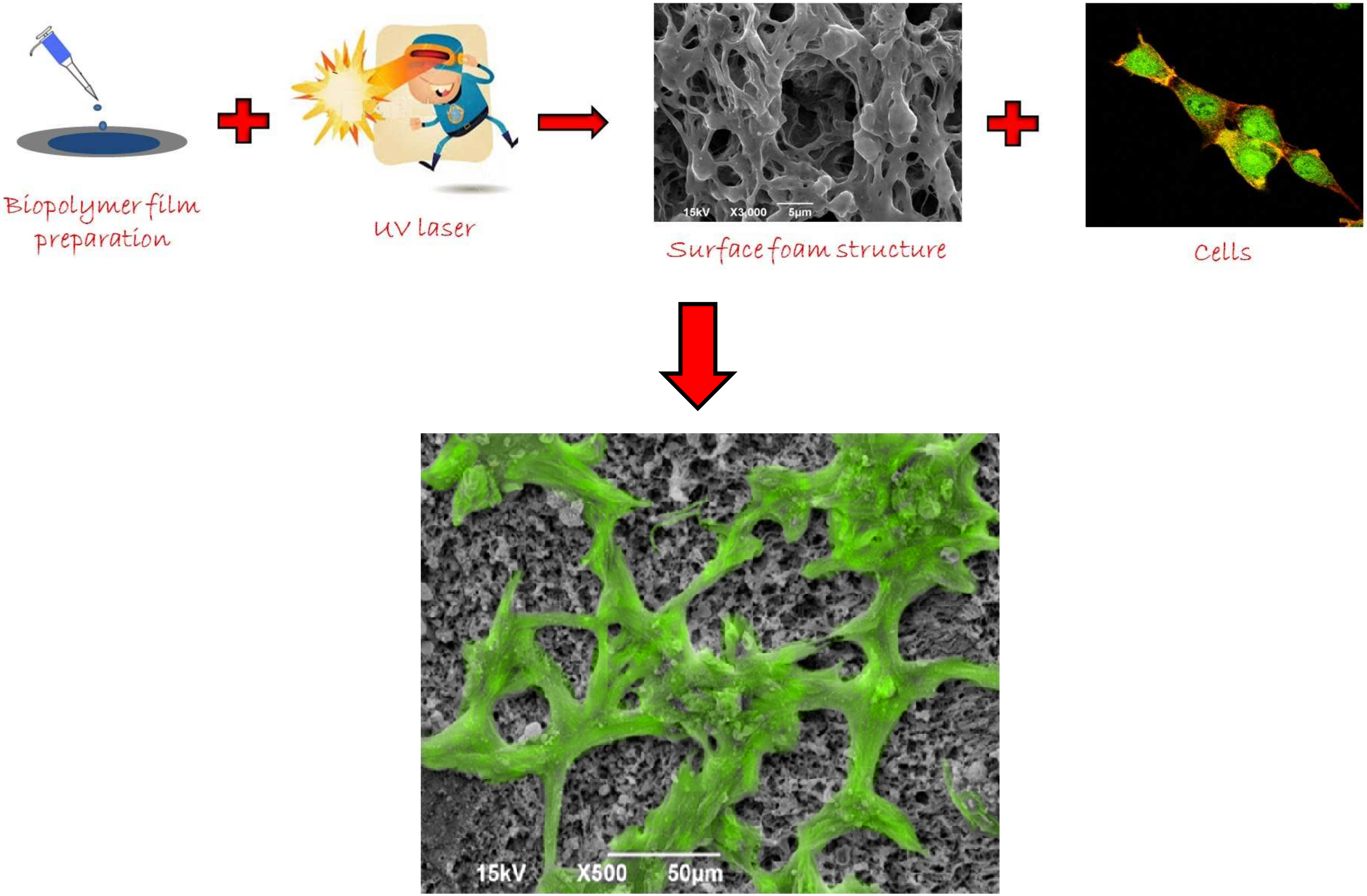
Laser based fabrication of biomimetic scaffolds

I. **Ultrafast laser micro/nano structuring**
   Hierarchical micro/nano structuring

II. **3-D nano-structuring using multi-photon polymerization**
   Sub-diffraction limit structuring

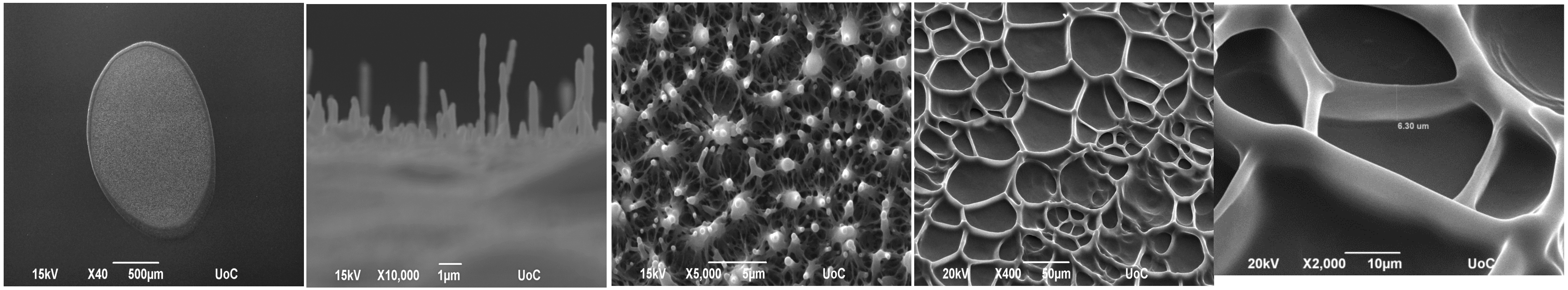
III. **Single pulse UV laser irradiation of biopolymers**
   Micro porous foam structuring on natural biopolymers

Process followed:

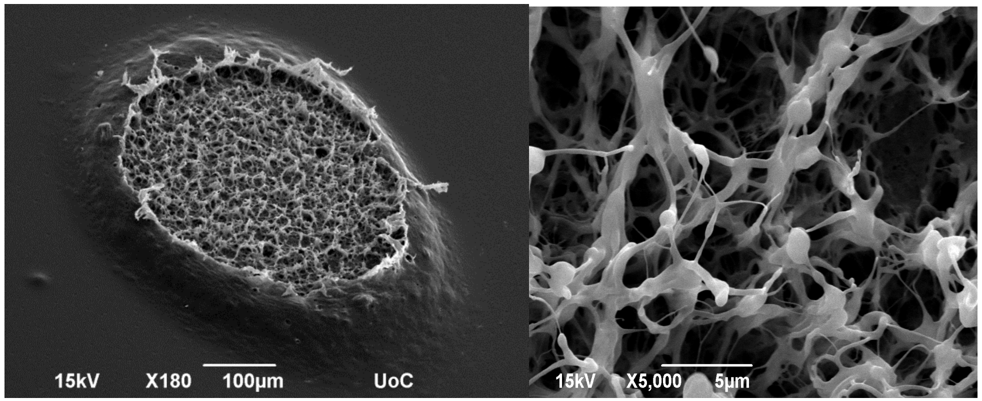


Biopolymer foam-like scaffolds casted on glass

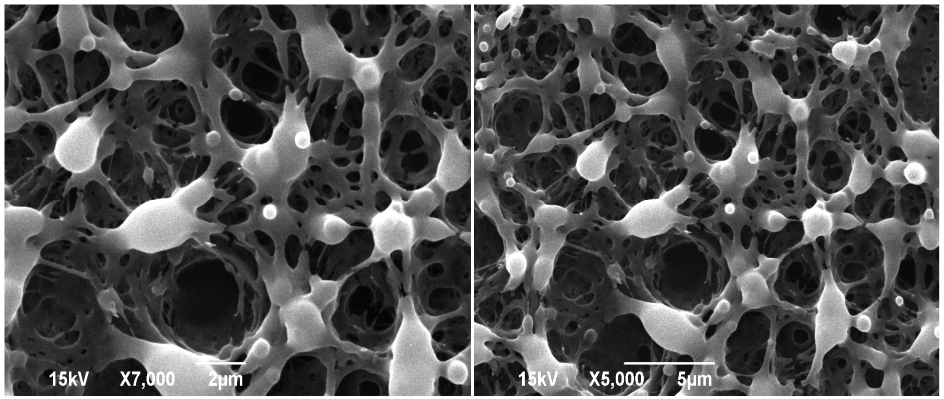
Gelatin



Chitosan

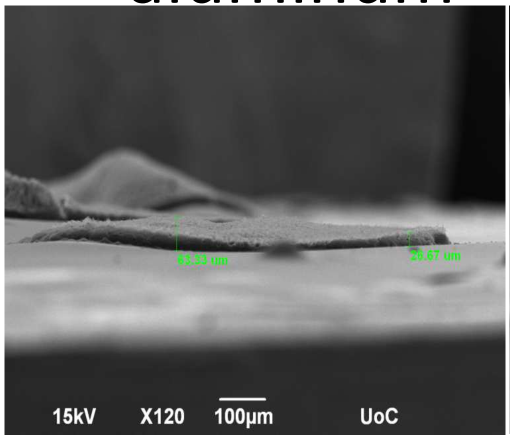


Collagen

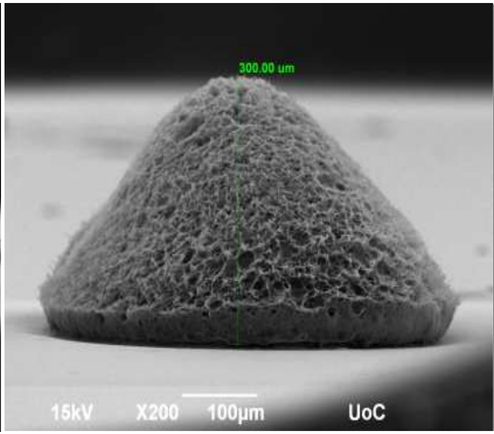


Chitosan foam-like scaffolds casted on different substrates:

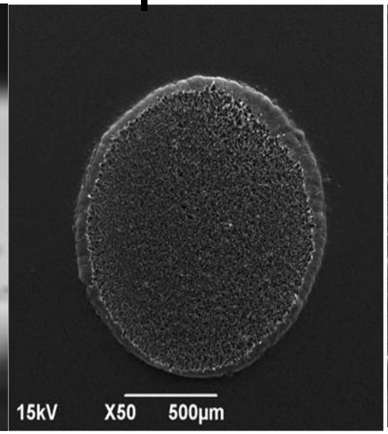
aluminum



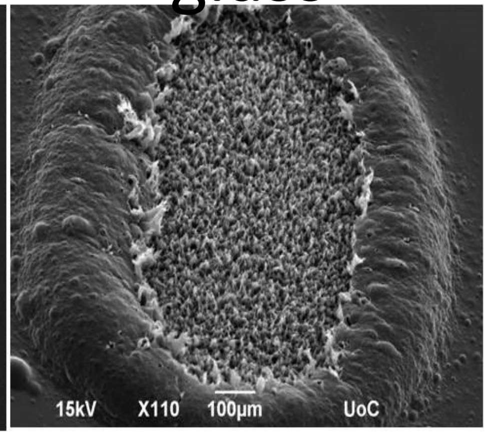
silicon



quartz



glass



Conclusion

The cell response (**outgrowth** & **differentiation**) was influenced by the substrate topography.

The micropatterned and 3D bridge-bearing substrates can support outgrowth and network formation of dissociated ganglion neurons.

There is a trend for **preferred outgrowth orientation** of Schwann cells.

⇩

a correlation between the geometrical characteristics of the topographical features of the surfaces and the (respective) cell responses

Thank you
for your attention !