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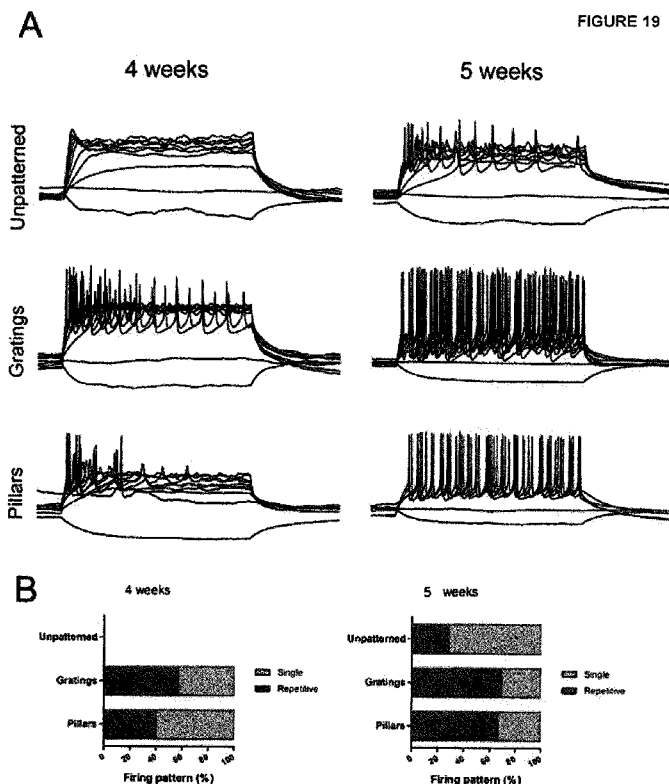
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(54) Title: NEUROGENESIS OF DOPAMINERGIC NEURONS



(57) Abstract: The invention relates to a method of enhancing the differentiation of cells into neurons, wherein the cells are selected from the group multipotent stem cells, pluripotent stem cells and somatic cells, comprising seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium; said substrates; a composite comprising at least one of said substrate, wells or chambers and either a plurality of said multipotent or pluripotent cells or a plurality of multipotent or pluripotent cell progeny, including cells differentiated therefrom; and a drug screening method involving the use of said composite.



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NEUROGENESIS OF DOPAMINERGIC NEURONS

FIELD OF THE INVENTION

The present invention relates to novel substrates that facilitate the differentiation of multipotent or pluripotent cells, such as stem cells or progenitor cells and somatic cells; wells or chambers
5 containing said substrates, particularly, but not exclusively, for culturing said cells; a method for the differentiation of multipotent, pluripotent or somatic cells involving use of said substrates, wells or chambers; a composite comprising at least one of said substrates, wells or chambers and either a plurality of said multipotent, pluripotent or somatic cells or a plurality of multipotent, pluripotent or somatic cell progeny, including cells differentiated therefrom; and a drug screening
10 method involving the use of said composite.

BACKGROUND OF THE INVENTION

Stem cell research could potentially impact the development of disease-modifying therapies, such as those designed to treat Parkinson's disease (PD), which has been hampered by the lack of predictive and progressive cellular models. Parkinson's disease, which affects more than
15 2% of the population over 65 years of age, and is associated with motor and cognitive deficits, has been attributed to the loss of midbrain dopaminergic (DA) neurons within the pars compacta of the substantia nigra [Damier P, et al., *Brain* 122: 1437-1448 (1999)]. The development of new *in vitro* models of the disease, such as using patient-derived pluripotent stem cells, would enable drugs against disease pathology to be screened more efficiently. These methods can then be
20 used to evaluate environmental and genetic factors implicated in PD, as well as elucidate the underlying biological mechanisms associated with this disease.

Although considerable progress has been made in deriving DA neuronal cells from stem cells, more work must be done to derive DA cells that are robust and reproducible in quality and quantity, and that can be enriched for selective subtypes of DA neurons. The desired cell
25 population must be clearly defined and stringently purified before stem cell-derived DA neurons can be used for future clinical applications. Deriving midbrain DA neurons *in vitro* requires several biochemical cues including a variety of growth factors and patterning neurotrophic factors to increase derivation efficiency [Chambers SM, et al., *Nat Biotechnol* 27: 275-280, (2009); Kriks S, et al., *Nature* 480: 547-551 (2011)]. However, this can be impractical and
30 expensive for large-scale production of neurons. Although methods have been devised using fewer growth factors [Erceg S, et al., *PLoS ONE* 3: e2122 (2008)], such methods yield lower

subtype specific neurons, requiring purification of the cell population, which further reduces the cell yield.

Interactions between cells and their mechanical microenvironment play important roles in determining cell fate and cell behaviour. Cell survival, proliferation and their propensity to differentiate into specific cell types are affected by extracellular biochemical and biophysical signals [Dingal P, Discher D. *Nat Mater* 13: 532-537 (2014)]. Accumulating evidence demonstrates that the topography of substrates that cells adhere to influence their response [Ankam S, et al., *Acta Biomater* 9: 4535-4545 (2013); Hoffman-Kim D, et al., *Annu Rev Biomed Eng* 12: 203-231 (2010); Koo S, et al., *Acta Biomater* 10: 1975-1984 (2014); Yim E, et al., *Biomaterials* 31: 1299-1306 (2010); Lim J, et al., *Biomacromolecules* 6: 3319-3327 (2005); Hamilton D, et al., *Cell Motil Cytoskeleton* 66: 260-271 (2009); Mahoney M, et al., *Biomaterials* 26: 771-778 (2005); Rajniecek A, McCaig C. *J Cell Sci* 110 (23): 2915-2924 (1997); Gerecht S, et al., *Biomaterials* 28: 4068-4077 (2007)]. In our previous work, we have shown that patterned nanostructures are able to induce neuronal marker expression in human mesenchymal stem cells [Yim E, et al., *Exp Cell Res* 313: 1820-1829 (2007)]. Neural stem cells are also influenced by nanoscale patterned substrates, which for instance, cause them to elongate along poly (L-lactic acid) fibres with significant outgrowth of neurites in the direction of the fibres [Yang F, et al., *Biomaterials* 26: 2603-2610 (2005)]. Similarly, other groups have shown that aligned topographical structures are able to promote differentiation and neurite alignment as well as influence cell maturation [Chew S, et al., *Biomaterials* 29: 653-661 (2008); Christopherson G, et al., *Biomaterials* 30: 556-564 (2009); Johansson F, et al., *Biomaterials* 27: 1251-1258 (2006)].

By taking into account biophysical influences in neuronal differentiation, other studies have shown that certain topographies can enhance differentiation of neural progenitors toward neural lineages [Moe AAK, et al., *Small* 8: 3050-3061 (2012); Migliorini E, et al., *Biotechnol Bioeng* 108: 2736-2746 (2011)]. Additional studies have also shown that topography can enhance neuronal differentiation of other stem cell types [Migliorini E, et al., *Biotechnol Bioeng* 108: 2736-2746 (2011); Pan F, et al., *Biomaterials* 34: 8131-8139 (2013); Yang K, et al. *ACS Applied Materials & Interfaces* 5: 10529-10540 (2013); Lee MR, et al., *Biomaterials* 31: 4360-4366 (2010); Chan LY, et al., *Biomaterials* 34: 382-392 (2013)]. However, differentiation of these stem cells into particular neuronal subtypes remains unexplored for specific applications, such as the treatment of PD.

Here, we demonstrate that certain topographies improve the efficiency of DA neuron derivation. We used an 18-pattern Multi-Architecture (MARC) chip with a combination of micro- or nano-patterned substrates to optimize and identify patterns or structures that can best improve the efficiency of neural progenitor cell differentiation into the midbrain DA neuronal subtype. We showed that, for neural progenitor cells, gratings with specific dimensions improve derivation as well as maturation of the DA neuron subtype, with an associated alignment of these neurons along the grating patterns. We also showed that, for pluripotent stem cells, a 2 stage method involving the use of a separate grating in each stage resulted in the production of morphologically and electrophysiologically mature dopaminergic neurons. We also show that the transdifferentiation of somatic cells into neuronal lineage can be significantly improved on patterned substrates. The results of this study thus reveal how topographical cues can be used to generate neurons, particularly DA neurons for cellular models of PD.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides a method of enhancing the differentiation of cells into neurons, wherein the cells are selected from the group multipotent stem cells, pluripotent stem cells and somatic cells, comprising seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium.

In a preferred embodiment the method of enhancing the differentiation of cells into neurons comprises two stages;

i) seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium, and

ii) removing the cells from the substrate of stage i) after a period of contact with medium and re-seeding the removed cells onto a substrate patterned with nano- and/or micro gratings or pillars, and contacting the cells with medium.

According to a further aspect of the invention there is provided a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected

from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, wherein the dimensions of the gratings are 250 nm to 2 μ m wide with 250 nm to 2 μ m spacing and 250 nm to 2 μ m height.

- 5 According to a further aspect of the invention there is provided the use of dopaminergic neurons produced according to any aspect of the invention for the treatment of Parkinson's disease.

According to a further aspect of the invention there is provided a method of treatment of Parkinson's disease, comprising administering to a subject in need thereof an efficacious amount of dopaminergic neurons produced according to any aspect of the invention.

- 10 According to a further aspect of the invention there is provided the use of dopaminergic neurons produced according to any aspect of the invention to screen for compounds with activity in ameliorating or treating Parkinson's disease, in vitro testing or drug screening related to Parkinson's disease or diseases related to dopaminergic neurons.

- According to a further aspect of the invention there is provided a method of testing compounds
15 for activity in ameliorating or treating Parkinson's disease, comprising the steps:

(a) contact dopaminergic neurons obtained according to any aspect of the invention with the test compound, and

- (b) quantitate the level of any one or more from the group comprising dopamine production, TH expression, Lmx1a expression, Foxa2 expression, PITX3 expression, MAP2 expression,
20 NURR1 expression, ALDH1a1 expression, SCN1a expression, VMAT2 expression, Pax6 expression and neuron complexity; and/or

(c) measure the electrophysiological profile of said neurons; and

- (d) compare the level quantitated in (b) and/or measured in (c) with the level in untreated dopaminergic neurons, wherein a difference in level of dopamine production, TH expression,
25 Lmx1a expression, Foxa2 expression PITX3 expression, MAP2 expression, NURR1 expression, ALDH1a1 expression, SCN1a expression, VMAT2 expression, Pax6 expression, neuron complexity and/or electrophysiological profile indicates the compound has Parkinson's disease-ameliorating activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Shows the dimensions, construction (A) and arrangement (B) of the MARC chip used to determine the effect of 3-dimensional topographies on cell differentiation.

Figure 2: Neural progenitor cells (passages 12-19) isolated from the hippocampus of 5-day old mice were seeded onto laminin-coated plates and characterized by their immune-reactivity against nestin and SOX2 (A) and GFAP (B, C), and their ability to differentiate into neurons and astrocytes.

Figure 3: Controlled differentiation of murine neural progenitor cells (mNPCs) to dopaminergic neurons on poly-dimethylsiloxane (PDMS). (A) Timeline of differentiation of mNPCs to dopaminergic neurons and the composition of the media for induction and patterning. (B) Scanning electron microscope images of the gratings of size 250nm with 250 nm pitch and 250 nm height, 2 μ m with 2 μ m width and 2 μ m height, and hierarchical grating of size 2 $\times$ 2 $\times$ 2 μ m gratings with hierarchical perpendicular 250 $\times$ 250 $\times$ 250 nm grating on the ridge (2 $\times$ 2 $\times$ 2 μ m gratings $\perp$ 250 $\times$ 250 $\times$ 250 nm lines) replicated with high fidelity onto PDMS substrates. No topographical features were observed on the unpatterned control.

Figure 4: Controlled differentiation of human induced pluripotent stem cells (iPSCs) to midbrain dopaminergic neurons on patterned poly-dimethylsiloxane (PDMS). (A) Timeline of differentiation of human iPSCs to dopaminergic neurons and the composition of the media at different stages. Modifications and optimization to the protocol based on dual SMAD inhibition method (Kirkeby A, et al. *Cell reports* 1: 703-714 (2012)) were made incorporating the use of topographical patterns for dopaminergic neuronal differentiation. The size of embryoid bodies was standardized on fabricated PDMS chambers consisting of microwells, whereupon they were harvested for a two-stage differentiation process on fabricated patterned chambers involving the expansion of neural progenitor cells (stage 1) and the terminal differentiation of neurons (stage 2). Concentrations of PLO/FN/Lam were optimized on the substrates for cell attachment. (B) Brightfield images of embryoid bodies in microwells and on patterned substrates at stage 1. Cells were observed to be aligned on the grating axis (arrow) at stage 1. Scale bar: 100 μ m (C) Immunofluorescence images of cells expressing tyrosine hydroxylase (TH), a dopaminergic neuron marker at stage 2 on patterned substrates. Scale bar: 50 μ m. (EB: embryoid body; NB: neurobasal medium; PLO: poly-L-ornithine; FN: fibronectin; Lam: laminin; SHH: sonic hedgehog; BDNF: brain-derived neurotrophic factor; GDNF: glial cell derived neurotrophic factor; TH: tyrosine hydroxylase; BF: brightfield).

Figure 5: Scanning electron microscopy images (left to right) of an unpatterned surface, gratings of size 2 μm with 2 μm width and 2 μm height, 250 μm with 250 nm pitch and 250 nm height and hierarchical gratings of size 2 μm with 250 nm perpendicular gratings.

Figure 6: Results comparing gene expression upon differentiation on patterned PDMS surfaces.

- 5 (A) TH is a marker of dopaminergic neurons and (B) PITX3 is a marker of nigral dopaminergic cells.

Figure 7: Screening with 18 pattern Multi-Architecture (MARC) chip. (A) Quantification and data analysis of GFAP+ (glial fibrillary acidic protein) (astrocytes) and TUJ1+ (β -III-tubulin) (neurons) cells on 18 pattern MARC chip. 2 μm ×2 μm ×2 μm gratings (1), 250 nm×250 nm×250 nm gratings
 10 (5), and 2×2×2 μm gratings $\perp$ 250×250×250 nm lines (6) produced significantly higher percentage of TUJ1-positive cells compared to the unpatterned substrate (n=3). * p <0.05, ** p <0.01 are compared to unpatterned control. # p <0.05, ## p <0.01 are compared between different patterns. (B) Quantification and data analysis of TH+ (tyrosine hydroxylase) (dopaminergic) cells on 18 pattern MARC chip. 2 μm ×2 μm ×2 μm gratings (1), 250 nm×250
 15 nm×250 nm gratings (5), and 2×2×2 μm gratings $\perp$ 250×250×250 nm lines (6) produced significantly higher percentage of TH-positive cells compared to the unpatterned substrate (n=3). ** p <0.01, *** p <0.001 are compared to unpatterned control. Comparison between all the patterns is tabulated in Supplementary Table 2. (C) Quantification and data analysis of TH-positive cells over TUJ1-positive cells on 18 pattern MARC chip (n=3). All data are represented as data $\pm$
 20 standard error mean (SEM) of three independent experiments. * p <0.05 compared to unpatterned control. # p <0.05, ## p <0.01 compared between different patterns.

Figure 8: Quantitative real-time PCR for Gene Expression Analysis. (A) Relative gene expression of tyrosine hydroxylase (TH) after 11 days of differentiation. (B) Relative gene expression of pituitary homeobox 3 (PITX3) after 19 days of differentiation.

25 **Figure 9:** Immunofluorescence staining of dopaminergic neuronal markers on the differentiated murine neural progenitor cells on PDMS 2×2×2 μm gratings, 250×250×250 nm gratings, hierarchical gratings, and unpatterned PDMS substrates. Neuronal markers tyrosine hydroxylase (TH), microtubule associated protein 2 (MAP2), nuclear receptor related protein 1 (NURR1), voltage gated sodium channels (SCN1a), and vesicular monoamine transporter 2 (VMAT2),
 30 aldehyde dehydrogenase 1 family member A1 (ALDH1a1), pituitary homeobox 3 (PITX3) in green. Nuclei were counter-stained with DAPI (blue). Arrows indicate the direction of the major grating axis.

Figure 10: Population counts of stained murine neural progenitor cells on single patterned or unpatterned PDMS substrates after differentiation. (A) Quantification and data analysis of TH-positive (dopaminergic) cells (n=3) after 19 day differentiation on 2µm×2µm×2µm gratings and unpatterned PDMS substrates. (B) Quantification and data analysis of MAP2+ (microtubule associated protein 2) cells (n=4) after 11 day differentiation on 2µm×2µm×2µm gratings and unpatterned PDMS substrates. (C) Quantification and data analysis of ALDH1a1+ (aldehyde dehydrogenase 1 family member A1) (dopaminergic precursor) cells on 2µm×2µm×2µm gratings, 250 nm×250 nm×250 nm gratings, and unpatterned PDMS substrates (n=3). (D) Quantification and data analysis of LMX1a+ (LIM homeobox transcription factor 1 alpha) (dopaminergic precursor) and PITX3+ (pituitary homeobox 3) (nigral neurons) cells on 2µm×2µm×2µm gratings, 250 nm ×250 nm×250 nm gratings, and unpatterned PDMS substrates (n=3). All data are represented as data ± SEM of n independent experiments. **p*<0.05 ***p*<0.01, ****p*<0.001 compared to unpatterned control. Scale bar, 50 µm.

Figure 11: Elongation and alignment of neurons on single patterned PDMS substrates. (A) Immunofluorescence staining of TUJ1 and GFAP in cells on 2×2×2 µm gratings, 250×250×250 nm gratings, hierarchical gratings (2×2×2 µm gratings ⊥ 250×250×250 nm lines), and unpatterned PDMS substrates. Cells were more elongated and aligned on the 2×2×2 µm and hierarchical gratings axis. Arrows indicate the direction of the major grating axis. Scale bar: upper panel 100 µm; bottom panel, 50 µm. (B) Neurons were more elongated when differentiated on 2×2×2 µm gratings and hierarchical gratings than unpatterned control. (C) Significantly more neurons were aligned on the grating axis on the 2×2×2 µm gratings and hierarchical gratings than on the 250×250×250 nm gratings. All data are represented as mean ± SEM and experiments were carried out in duplicate or more with over 200 cells measured on each sample.

Figure 12: Stiffness measurement by atomic force microscopy (AFM) showed the elastic modulus of PDMS (10:1 base/curing ratio) and TCPS to be 3.27 ± 0.133 MPa and 2149 ± 285 MPa respectively, indicating a considerable difference in stiffness between these two substrates.

Figure 13: Population counts of stained cells on single patterned or unpatterned TCPS substrates after 11 days of differentiation. (A) Immunofluorescence staining of TUJ1, GFAP and MAP2 in cells on 2×2×2 µm gratings, 250×250×250 nm gratings, and unpatterned TCPS substrates. Arrows indicate the direction of the grating axis. Scale bar, 100 µm. (B) 2×2×2 µm gratings generated higher neuron-to-astrocyte ratios than unpatterned control based on staining

of TUJ1 (neurons) and GFAP (astrocytes). Data are represented as data $\pm$ SEM of at least two independent experiments. (C) Quantification and data analysis of MAP2-positive (mature neuron) cells on $2\mu\text{m}\times 2\mu\text{m}\times 2\mu\text{m}$ gratings, $250\text{ nm}\times 250\text{ nm}\times 250\text{ nm}$ gratings, and unpatterned TCPS substrates. Both gratings produced more MAP2+ cells than unpatterned control. Data are represented as data $\pm$ SEM of at least three independent experiments. $*p<0.05$, $**p<0.01$ compared to unpatterned control.

Figure 14: Immunostaining of dopaminergic neuronal markers on human iPSC-derived cells on unpatterned, gratings, and pillars PDMS substrates. Beta-III tubulin (TUJ1), microtubule associated protein 2 (MAP2), pituitary homeobox 3 (PITX3), LIM homeobox transcription factor 1 alpha (LMX1a), forkhead box protein A2 (FOXA2) were all robustly expressed in patterned substrates.

Figure 15: Quantification and data analysis of stained cells on patterned and unpatterned substrates after 21 day differentiation. (A) Quantification and data analysis of TUJ1-positive (neuronal) cells after 21 day differentiation on unpatterned, gratings and pillars PDMS substrates. Both gratings and pillars have higher percentages of TUJ1-positive cells than unpatterned ($n=5$). (B) Quantification and data analysis of TH-positive (dopaminergic) cells after 21 day differentiation on unpatterned, gratings and pillars PDMS substrates ($n=5$). (C) Percentage of TH-positive cells normalized on unpatterned substrates for each experiment. Gratings have about two-fold increase in TH-positive cells than unpatterned ($n=5$). (D) Quantification and data analysis of TH/TUJ1 cells on unpatterned, gratings, and pillars PDMS substrates ($n=5$). (E) Quantification and data analysis of FOXA2-positive (midbrain DA) cells on unpatterned, gratings, and pillars PDMS substrates. Gratings and pillars have more cells expressing FOXA2 than unpatterned ($n=3$). All data are represented as data $\pm$ SEM of n independent experiments. At least five fields of view were taken for analysis and over 200-1000 cells were counted for each sample. $*p<0.05$ $**p<0.01$ compared to unpatterned control. Scale bar, $100\text{ }\mu\text{m}$. (TUJ1: beta-III tubulin; TH: tyrosine hydroxylase; FOXA2: forkhead box A2).

Figure 16: Morphology of TH neurons on patterned and unpatterned substrates after 21 day differentiation. (A) Representative images of human iPSC-derived TH neurons on unpatterned, gratings and pillared PDMS substrates. Images were digitally stitched from overlapping fields of view to capture the entire length of neurites. Cells on gratings were aligned on the gratings axis (arrows). Scale bar: $100\text{ }\mu\text{m}$ (B) Average neurite length per neuron. TH neurons were more elongated when differentiated on gratings than pillars and unpatterned control. (C) Number of

branch points per neuron (D) Sholl analysis. TH neurons have significantly more branching and increased neuronal complexity when differentiated on pillars than gratings and unpatterned control. (E) Number of terminals per neuron is significantly greater when differentiated on pillars. (F) Graph shows percentage of spontaneous postsynaptic current is higher in TH +ve neurons differentiated on pillars. (G) Representative trace shows spontaneous synaptic activity (arrowhead) can be detected as early as 4 weeks post differentiation. Scale bar: 10 pA (vertical) and 2 s (horizontal). Inset (red box) scale bar: 10 pA (vertical) and 0.2 s (horizontal). All data are represented as data $\pm$ SEM of three independent experiments with over 30 TH +ve neurons analyzed on each pattern. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

Figure 17: Electrophysiology properties of human iPSC-derived neurons on unpatterned, gratings, and pillars PDMS substrates. (A) Shows representative tracings of firing patterns on substrates at 4 and 5 weeks post differentiation. Neurons were capable of firing repetitive action potential as a response to current injection as early as 4 weeks post differentiation. (B) Percentage of differentiated neurons capable of repetitive firing at 5 weeks post differentiation. More neurons on pillars were capable of repetitive firing than when on unpatterned and gratings. Five to nine neurons were recorded from three experiments per condition.

Figure 18: Immunostaining of dopaminergic neuronal markers on human iPSC-derived cells from Parkinson's disease patient with LRRK2 mutation on unpatterned, gratings, and pillars PDMS substrates. TUJ1, TH and LMX1a were robustly expressed indicating patient-derived iPSC cells can be successfully differentiated to midbrain dopaminergic neurons on patterned substrates.

Figure 19: Electrophysiology properties of human induced pluripotent stem cell (iPSC)-derived cells from PD patient with LRRK2 mutation on unpatterned, gratings, and pillars PDMS substrates. (A) Representative tracings of firing patterns on substrates at 4 and 5 weeks post differentiation. Neurons were capable of firing a repetitive action potential as a response to current injection as early as 4 weeks post differentiation. (B) Shows the percentage of differentiated cells capable of repetitive firing at 5 weeks post differentiation. More neurons on gratings and pillars were capable of repetitive firing than on unpatterned PDMS.

DETAILED DESCRIPTION OF THE INVENTIONDefinitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

- 5 Reference herein to a biologically inert polymer is reference to a polymer that is chemically inactive, at least during the period of use for the differentiation of multipotent or pluripotent cells, thus the invention also includes polymers that, after use, are biodegradable.

Further, reference herein to multipotent or pluripotent cells, is reference to stem cells, progenitor cells and induced pluripotent stem cells.

- 10 As used herein, reference to a "grating" is reference to a micro or nanoscale structure of a series of parallel beams with crests and troughs resulting in three dimensional grooves of specific space(s) between the walls of each groove, height(s) of the wall of the groove and width(s) of the walls of the groove.

- 15 As used herein, reference to a "hierarchical grating" is reference to a grating with an overlay of set(s) of micro and/or nanoscale three dimensional structures with dimensions smaller than that of the first resulting in a grating with indentations or undulations on its walls and/or the width of its walls and/or within the space between the walls.

As used herein, reference to "pillars" is reference to a series of microscale or nanoscale upright shafts or structures relatively slender in proportion to their height, and of any shape in section

- 20 In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the term "comprising" as used in the context of the invention refers to where the various components, ingredients, or steps, can be conjointly employed in practicing the present invention. Accordingly, the term "comprising" encompasses the more restrictive terms "consisting essentially of" and "consisting of." word "comprises", or variations such as "comprised" or
25 "comprising" is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

All references, including any patent or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art.

- 5 Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

A person skilled in the art will appreciate that the present invention may be practiced without undue experimentation according to the method given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology
10 and molecular biology text books.

According to a preferred aspect, the present invention provides a method of enhancing the differentiation of cells into neurons, wherein the cells are selected from the group multipotent stem cells, pluripotent stem cells and somatic cells, comprising seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-
15 grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium.

Preferably, the dimensions of the gratings are 250 nm to 2 μ m wide with 250 nm to 2 μ m spacing and 250 nm to 2 μ m height, and the hierarchical secondary structures are gratings with
20 dimensions of 100 nm to 250 nm wide with 100 nm to 250 nm spacing and 100 nm to 250 nm height; and the pillars are 250 nm to 40 μ m diameter with a 1 μ m to 15 μ m pitch. It is preferred that the pillars are 0.5 μ m to 10 μ m diameter, more preferably 0.5 μ m to 5 μ m, with a 5 μ m to 12 μ m pitch; even more preferably the pillars are 1 μ m to 2 μ m in diameter with an 8 μ m to 12 μ m pitch. It would be understood that intermediate sizes not particularly specified herein may be
25 suitable according to the invention. In particular, it is preferred that the gratings according to any aspect of the invention are parallel. It is also preferred that the hierarchical secondary gratings according to any aspect of the invention are perpendicular to the longitudinal axis of the gratings.

In another preferred embodiment, the gratings have an aspect ratio of 1 (grating width: spacing:
30 height of 1:1:1).

In a preferred embodiment, the method of enhancing the differentiation of cells into neurons further comprises the steps;

- a) maintaining the seeded cells in medium;
- b) inducing neuronal lineage phenotype by withdrawal of growth factors and/or inhibitors;
- 5 c) replacing the medium of b) with neural patterning medium.

In a preferred embodiment the method of enhancing the differentiation of cells into neurons comprises two stages;

- 10 i) seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium, and
- 15 ii) removing the cells from the substrate of stage i) after a period of contact with medium and re-seeding the removed cells onto a substrate patterned with nano- and/or micro gratings or pillars, and contacting the cells with medium.

In this two stage method, pluripotent stem cells such as, for example, induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) may be seeded onto a substrate patterned with gratings in step i) in order to increase lineage commitment towards midbrain dopaminergic neurons. After a period of culture on the substrate, the cells may be removed from the gratings and re-seeded onto a substrate patterned with pillars to promote neurite branching and a more differentiated morphology with an increased capability of firing repetitive action potentials. These cells also showed increased spontaneous postsynaptic activity and better electrophysiology profile. See, for example, Figures 17 and 19 herein.

25 In a preferred embodiment of the invention, there is provided a two stage method of enhancing the differentiation of embryoid body (EB) cells, derived from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), into dopaminergic neurons comprising;

- i) seeding dissociated embryoid body (EB) cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with parallel

grating structures, and contacting the cells with medium comprising SMAD inhibitor, Noggin, sonic hedgehog (SHH) and CT99021; and

ii) removing the cells from the substrate of stage i) after a period of contact with said medium and re-seeding the removed cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with gratings or pillars, and contacting the cells with medium comprising BDNF, GDNF and Ascorbic Acid; and adding DAPT to the medium at day 4.

In a more preferred embodiment of the invention, there is provided a two stage method of enhancing the differentiation of embryoid body (EB) cells, derived from human induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), into dopaminergic neurons comprising;

i) seeding dissociated embryoid body (EB) cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with 2 μ m ridge by 2 μ m space by 2 μ m depth parallel grating structures, and contacting the cells with medium comprising SMAD inhibitor SB431542, Noggin, SHH and CT99021 to promote initial lineage commitment of midbrain DA neurons; and

ii) removing the cells from the substrate of stage i) after a period of contact with said medium and re-seeding the removed cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with 2 μ m diameter pillars with 2 μ m height, and contacting the cells with medium comprising BDNF, GDNF and Ascorbic Acid for 3 days; and

adding DAPT to the medium at day 4 for at least another 7 days to promote differentiation into functional midbrain dopaminergic neurons.

In another preferred embodiment, the neural patterning medium in c) comprises Neurobasal and DMEM/F12 in 1:1 ratio, 1x B27 supplement, 0.25x N2 supplement, 100ng/ml FGF-8, 200ng/ml Sonic Hedgehog and 1 μ g/ml laminin or, if the cells are pluripotent stem cells, the medium in c) comprises BDNF (20 ng/ml), GDNF (10 ng/ml) and ascorbic acid (200 μ M) for 3 days, and then *N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) (2.5 μ M) is added to the medium from day 4 of stage 2.

According to any aspect of the present invention, the embryoid bodies are preferably formed in microwells having a diameter of about 500 μm to 800 μm . In a preferred embodiment, the embryoid bodies are grown to about 300 μm in diameter before being dissociated and seeded onto substrate in stage (i).

- 5 According to any aspect of the present invention, the seeded cells are preferably contacted with the neural patterning medium for at least 5 to 30 days, more preferably for at least 7 to 19 days.

Preferably in the two stage method the substrate is PDMS.

DAPT is an inhibitor of γ -secretase, resulting in promotion of neuronal commitment. It would be understood that other γ -secretase inhibitors may be useful in the methods of the invention.

- 10 In a preferred embodiment of the method, the seeded cells are differentiated into dopaminergic neurons, which typically express tyrosine hydroxylase (TH). More preferably, the cells are differentiated into TH⁺ neurons which express substantia nigra dopaminergic neuron markers (PITX3+) and/or midbrain dopaminergic neuron markers (FOXA2, NURR1 and LMX1a).

- 15 In another aspect of the invention, the cells are somatic cells and they are trans-differentiated by non-viral transfection of one or more transcription factors. Preferably, the somatic cells are fibroblasts. The somatic cells may be derived from a vertebrate. Preferably, the cells are derived from a mammal, such as a mouse. More preferably, the somatic cells are derived from a human. The human may have Parkinson's disease.

- 20 In a preferred embodiment of the invention, the hierarchical secondary structures are selected from the group comprising gratings, pillars, columns and lens.

- The use of a substrate comprising gratings or hierarchical gratings during transdifferentiation of somatic cells increase the proportion of cells that are differentiated into neurons. For example, as shown herein, the proportion of fibroblasts that can be transdifferentiated into TUJ1⁺ cells is enhanced if they are plated onto gratings, particularly advantageously on hierarchical gratings
25 when compared to those plated onto unpatterned substrate. Preferably, hierarchical gratings, of size 2 μm with 250 nm perpendicular gratings, such as those shown in Figure 5 herein may be used.

- Somatic cells that have been transdifferentiated into neurons on a patterned substrate as described supra may be then subjected to a second stage of differentiation on a second
30 patterned substrate as described herein.

In a preferred embodiment, the substrate comprises a biologically inert polymer. The said substrate may be made from a silicone compound such as a silicone oil (polymerized siloxane), an example of which would be polydimethylsiloxane (PDMS). PDMS is the most widely used silicon-based organic polymer, it is optically clear, inert, non-toxic and non-flammable. Moreover, it is viscoelastic in nature, which complements the viscoelastic nature of cells. It does not swell with the addition of aqueous media, so it can be used without deformations. PDMS is a polymer that can change stiffness by simply changing the amount of the curing agent used. This means the invention provides a specified topography that can be used with the flexibility of various stiffness ranges.

However, those skilled in the art will appreciate that while PDMS is one of the preferred materials for the manufacture of our substrate other polymers that are chemically inert during the period of differentiation or use and which possess and one or more of the following characteristics, including any combination thereof, may be used: clear, inert, non-toxic, non-flammable, does not swell on exposure to aqueous media, it is an easy material to work, and it can change stiffness by simply changing the way it is made.

In an alternative embodiment of the invention said substrate is made from polystyrene, ideally tissue culture polystyrene (TCPS). The incorporation of nano-topography on tissue culture polystyrene, ideally, but not exclusively, with the help of nano-imprint lithography, allows for the use of this vastly accepted biomaterial in a novel way for the differentiation of multipotent or pluripotent cells and so allows for the generation of, for example, dopaminergic neurons.

According to a preferred embodiment of the invention, the substrate is selected from the group comprising polymethylsiloxane (PDMS) and tissue culture polystyrene (TCPS). According to a preferred embodiment of the invention, the PDMS grating structures are produced by soft lithography and the TCPS grating structures are produced by heat embossing.

In another preferred embodiment, the substrate is coated with extracellular matrix proteins prior to seeding. Preferably, the extracellular matrix proteins are selected from the group comprising poly-L-ornithine, fibronectin and laminin. One or more of these matrix proteins may be used for coating the substrate, and the particular repertoire used may depend on the type of cells to be differentiated, or the type of differentiated cells required. For example, as shown herein, mNPCs may be seeded onto substrate coated with laminin for neural induction and differentiation, whereas human iPSCs may be seeded onto substrate coated with laminin, fibronectin and poly-L-ornithine in the two stage method described. As herein disclosed, the amount of laminin used

to coat substrates for differentiation of mNPCs may be 5 µg/ml - 50 µg/ml. As herein disclosed, the amount of laminin, fibronectin and poly-L-ornithine used to coat substrates for differentiation of iPSCs may be 5 µg/ml - 50 µg/ml, 5 µg/ml - 50 µg/ml and 33 µg/ml - 100 µg/ml, respectively. Preferably, the amount of laminin, fibronectin and poly-L-ornithine is 20 µg/ml, 20 µg/ml and 33 µg/ml, respectively. It would be understood by the person skilled in the art that the amounts of extracellular matrix proteins to coat the substrate can vary from the stated amounts and still be effective, and determined without undue experimentation. Moreover, it would be understood that additional extracellular matrix proteins may be used with those specifically described herein.

According to a further aspect of the invention there is provided a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, wherein the dimensions of the gratings are 250 nm to 2 µm wide with 250 nm to 2 µm spacing and 250 nm to 2 µm height. Preferably the nano- and/or micro-grating structures are hierarchical gratings.

In a preferred embodiment of the substrate, the hierarchical secondary structures are selected from the group comprising gratings, pillars, columns and lens. More preferably the secondary structures are gratings or pillars. More particularly, the secondary structures are gratings.

More preferably, the hierarchical secondary structures are gratings with dimensions of 100 nm to 250 nm wide with 100 nm to 250 nm spacing and 100 nm to 250 nm height. Preferably the hierarchical secondary structure gratings are parallel.

In another preferred embodiment of the substrate, the hierarchical secondary structure gratings are perpendicular to the longitudinal axis of the gratings. Hierarchical gratings of this form have been shown herein to significantly increase the yields of subtype-specific and region-specific dopaminergic neurons compared to unpatterned substrates and gratings without secondary structure. In addition, we have found that the hierarchical gratings promote neuron differentiation and maturation as well as transdifferentiation.

More preferably still, the gratings have an aspect ratio of 1 (grating width: spacing: height of 1:1:1).

According to a further aspect of the invention there is provided the use of dopaminergic neurons produced according to any aspect of the invention for the treatment of Parkinson's disease.

According to a further aspect of the invention there is provided a method of treatment of Parkinson's disease, comprising administering to a subject in need thereof an efficacious amount of dopaminergic neurons produced according to any aspect of the invention. The subject may be a vertebrate. Preferably the subject is a mammal, such as a mouse. More particularly the subject is a human.

According to a further aspect of the invention there is provided the use of dopaminergic neurons produced according to any aspect of the invention to screen for compounds with activity in ameliorating or treating Parkinson's disease, in vitro testing or drug screening related to Parkinson's disease or diseases related to dopaminergic neurons.

According to a further aspect of the invention there is provided a method of testing compounds for activity in ameliorating or treating Parkinson's disease, comprising the steps:

(a) contact dopaminergic neurons obtained according to any any aspect of the invention with the test compound, and

(b) quantitate the level of any one or more from the group comprising dopamine production, TH expression, Lmx1a expression, Foxa2 expression PITX3 expression, MAP2 expression, NURR1 expression, ALDH1a1 expression, SCN1a expression, VMAT2 expression, Pax6 expression and neuron complexity; and/or

(c) measure the electrophysiological profile of said neurons; and

(d) compare the level quantitated in (b) and/or measured in (c) with the level in untreated dopaminergic neurons, wherein a difference in level of dopamine production, TH expression, Lmx1a expression, Foxa2 expression PITX3 expression, MAP2 expression, NURR1 expression, ALDH1a1 expression, SCN1a expression, VMAT2 expression, Pax6 expression, neuron complexity and/or electrophysiological profile indicates the compound has Parkinson's disease-ameliorating activity.

It would be understood by the skilled person that other markers or characteristics of dopaminergic neuron function may be suitable to quantitate in the aforesaid method of testing.

In a preferred embodiment of any aspect of the invention, the gratings are 2 μm wide with 2 μm spacing and 2 μm height, the hierarchical secondary gratings are 250 nm wide with 250 nm spacing and 250 nm height and are perpendicular to the longitudinal axis of said gratings and the pillars are 1 μm .

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (2001).

5 **Materials and Methods**

Fabrication of MARC chip

The Multi-Architecture (MARC) chip is a versatile customizable topography microarray with a size of 2.2 cm × 2.2 cm which can incorporate different topographies [Moe AAK, et al., *Small* 8: 3050-3061 (2012)]. Each of the different topographies has a field area of approximately 4 mm².

- 10 The MARC chip used in this study consists of 18 distinct surface topographies of 6 × 6 array in duplicates and an unpatterned polycarbonate film, serving as the unpatterned control field. The dimensions, construction and arrangement of the MARC chip are presented in Table 1 and Figure 1. In the texts henceforth, we would use the codes labelled in Table 1 when referring to the topographies.

15 Fabrication and replication of PDMS and TCPS substrates

- Polydimethylsiloxane (PDMS) substrates were fabricated using the Sylgard 184 Silicone Elastomer kit (Dow Corning, Michigan, USA) via soft lithography. The silicon molds used consist of the 18-pattern MARC chip with the patterns as listed in Table 1. The Sylgard 184 polymer (PDMS) was mixed with the accompanying curing agent in a 10:1 ratio and desiccated under vacuum for 30 min. The mixture was then poured into the silicon molds or unpatterned culture dishes, desiccated for another 30 min and placed into a 70°C oven to cure for 2 hours before demolding. On the other hand, the tissue culture polystyrene (TCPS) replicas were prepared by heat embossing.
- 20

TABLE 1: List of multi-architectural patterns on the MARC chip replicas used in the murine neural progenitor cell (mNPC) culture.

Category	No.	Pattern description
Gratings	1	2 μm gratings x 2 μm spacing x 2 μm height
	2	1 μm gratings x 2 μm spacing x 2 μm height
	3	2 μm gratings x 1 μm spacing x 1 μm height
	4	250 nm gratings x 250 nm spacing x 150 nm height
	5	250 nm gratings x 250 nm spacing x 250 nm height
Hierarchical gratings	6	2 μm gratings x 2 μm space & perpendicular 250 nm grating/250 nm spacing
	7	2 μm gratings x 2 μm space & parallel 250 nm grating/250 nm spacing
	8	2 μm gratings x 2 μm space & 250 nm dimple overlay
Pillars	9	2 μm diameter pillars with 10 μm pitch
	10	500 nm diameter pillars with 10 μm pitch
	11	130 nm diameter pillars with 400 nm pitch
	12	250 nm diameter pillars with 500 nm pitch
Wells	13	1 μm diameter wells with 8 μm pitch
Microlens	14	1 μm pitch microlens (concave)
	15	1 μm pitch microlens (convex)
	16	1.8 μm diameter with 2 μm pitch microlens (concave)
	17	1.8 μm diameter with 2 μm pitch microlens (convex)
Motheye	18	Motheye with 270 nm pitch

Preparation of substrates for murine neural progenitor cell (mNPC) culture

- 5 The PDMS or TCPS substrates were cut to fit into the culture plates and air-plasma treated (Harrick Plasma, Ithaca, NY, USA) for 120 seconds at 29.6 W. After washing in 100% ethanol, the substrates were placed under ultraviolet light for 40 min and coated with 33 $\mu\text{g}/\text{ml}$ poly-L-ornithine solution (Sigma-Aldrich, Missouri, USA) overnight. The substrates were then washed twice with sterile water and coated with 20 $\mu\text{g}/\text{ml}$ laminin (Life Technologies, California, USA)
- 10 overnight for controlled differentiation of mNPC. After each coating, vacuum degassing was applied for a short period to force the extracellular matrix proteins into the patterns.

Culture of murine neural progenitor cells (mNPCs)

The mNPCs (passages 12-19) were isolated from the hippocampus of 5-day old mice [20] and maintained on 8 µg/ml of laminin (Life Technologies) in a neural progenitor expansion medium. This medium contained Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F12 (Biological Industries, Israel) in a 1:1 ratio, 1× N2 supplements (Life Technologies) and 1× penicillin-streptomycin (Caisson Biotech, Texas, USA). 20 ng/ml basic fibroblast growth factor (bFGF) (Life Technologies) and 20 ng/ml epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA) were supplemented daily, with a complete medium change every two days. Upon achieving 80-90% confluence, cell passaging was performed by detaching with Accutase (Innovative Cell Technologies Inc, California, USA) and incubating at 37°C for 3 min. The cell suspension was centrifuged at 1100 rpm for 5 min and seeded onto laminin-coated plates at an approximately 1:3 ratio. Primary mNPCs were characterized by their immune-reactivity against nestin and SOX2, and their ability to differentiate into neurons and astrocytes (Figure 2). All procedures and care of animals were in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

Controlled differentiation of mNPCs into midbrain dopaminergic neurons

The mNPCs were seeded at 15,000 cells per cm² on the PDMS or TCPS substrates in the neural progenitor expansion medium. After allowing attachment of cells for 24 hours, the growth factors were withdrawn to induce differentiation into neuronal lineage. The medium was then changed to neural induction medium containing DMEM/F12, 1× N2 supplement, 1× penicillin-streptomycin solution, 5 ng/ml bFGF (Life Technologies) and 1 µg/ml laminin. Half of the medium was replaced every two days. On the 7th day of culture, the medium was changed to neural patterning medium containing Neurobasal (Life Technologies) and DMEM/F12 in 1:1 ratio, 1× B27 supplement (Life Technologies), 0.25× N2 supplement and 1× penicillin-streptomycin. The medium was supplemented with 100 ng/ml fibroblast growth factor 8 (FGF-8) (Sigma-Aldrich), 200 ng/ml recombinant Sonic Hedgehog (SHH) (R&D Systems) as well as 1 µg/ml laminin, and was replaced every two days. Differentiation was halted on the 11th or 19th day of culture. A graphical representation of the protocol is shown in Figure 3.

Controlled differentiation of human neural progenitor cells on substrate topography

The preparation of patterned substrates was as described above.

Human iPSCs or human ESCs derived from a normal subject or a PD patient were cultured using a feeder-free protocol. The pluripotent stem cells were expanded on Matrigel (Life Technologies) coated polystyrene culture plates with mTeSR 1 medium (Stemcell Technologies). Embryoid bodies (EBs) were formed using microwells (AggreWell, Stemcell Technologies), or other possible EB formation methodologies. Typically 1.5×10^6 cells are needed for a single microwell chamber. EB medium (DMEM/F-12 (24.25ml), Neurobasal medium (24.25ml), 1x N2 supplement, 1x B27 supplement and L-glutamine in 50 ml total) was supplemented with SB431542 (10 μ M) (Cellagen Technology), Noggin (200 ng/ml) (R&D Systems), SHH-C24II (200 ng/ml) (R&D Systems), CT99021 (0.8 μ M) (Cellagen Technology) and Y-27632 (10 μ M) (Tocris Bioscience) to each well of the microwell chambers that will be used. The EBs were incubated at 37°C with 5% CO₂ and 95% humidity for 4 days with a daily medium change. Aggregates formed were dislodged by carefully drawing up the medium and firmly pipette it back into the middle and around the wells. Dissociated EBs cells were seeded on 2 μ m ridge by 2 μ m space by 2 μ m depth grating pattern or unpatterned controls with poly-L-Ornithine (33 μ g/ml) (Sigma-Aldrich), fibronectin (20 μ g/ml) (Biological Industries) and laminin (20 μ g/ml) (Life Technologies) coating, for 7 days in supplemented media with dual SMAD inhibitor (SB431542 (10 μ M) (Cellagen Technology), Noggin (200 ng/ml) (R&D Systems), SHH-C24II (200 ng/ml) (R&D Systems), CT99021 (0.8 μ M) (Cellagen Technology) (Chamber S et al. Nat Biotechnol. 2009 Mar; 27(3): 275–280). Daily supplemented media changes were performed for the next 4 days followed by media change without supplements for another 2 days.

Terminal differentiation to dopaminergic neurons on substrate topography

The neural progenitor cells were then detached using Accutase (Stem Cell Technologies) and re-plated on either 2 μ m ridge by 2 μ m space by 2 μ m depth grating pattern, 1 μ m pillars or unpatterned controls with poly-L-Ornithine (33 μ g/ml) (Sigma-Aldrich), fibronectin (20 μ g/ml) (Biological Industries) and laminin (20 μ g/ml) (Life Technologies) coating. Daily media change was performed in the presence of BDNF (20 ng/ml) (Life Technologies) / GDNF (10 ng/ml) (Life Technologies) / ascorbic acid (200 μ M) (Sigma-Aldrich) for 3 days followed by the addition of DAPT (2.5 μ M) (Tocris Bioscience) for at least another 7 days (Figure 4). Differentiation can be terminated starting from day 21 for downstream applications.

Non-viral direct transdifferentiation of somatic cells to neuronal cells

The preparation of patterned substrates was described above.

Molecular cloning and plasmid purification.

As published in Adler A and Grigsby C et al., (*Molecular Therapy Nucleic Acids* 1: e32; doi:10.1038/mtna.2012.25 (2012), pmax-Brn2 (4,154 bp), pmax-Ascl1 (3,497 bp), and pmax-Myt1l (6,359 bp), which express the mouse transcription factors Brn2, Ascl1, and Myt1l, respectively, under control of the CMV promoter, were generated by first excising the GFP coding sequence from pmax-GFP (3486 bp; Amaxa, Cologne, Germany) with SacI digestion, blunting by DNA polymerase I Klenow fragment, NheI-HF digestion (NEB, Ipswich, MA), and gel extraction (QIAquick Gel Extraction Kit; QIAGEN, Hilden, Germany).

Then, Brn2, Ascl1, and Myt1l inserts were prepared by digestion of the lentiviral vectors Tet-O-FUW-Brn2 (Addgene 27151), Tet-O-FUW-Ascl1 (Addgene 27150), and Tet-O-FUW-Myt1l (Addgene 27152) with EcoRV and NheI-HF (NEB) and gel extraction, and were subsequently ligated into the empty pmax vector with T4 DNA ligase (NEB). When used together, these three plasmids are abbreviated as pmax-BAM. pUNO1-mAscl1 (3,892 bp; InvivoGen, San Diego, CA) and pUNO1-mMyt1lb (6,744 bp; InvivoGen), expressing mouse Ascl1 and Myt1l under control of the EF1 α /HTLV promoter, were used in conjunction with pmax-Brn2, and when used together are abbreviated as pUNO-AM/pmax-B. Plasmids were propagated in *Escherichia coli* DH5 α (Invitrogen, Carlsbad, CA) and purified with EndoFree Plasmid Mega and Maxi kits (QIAGEN). Plasmid DNA concentrations were quantified by measurement of absorbance at 260 nm with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

Non-viral transfection

While the Brn2, Ascl1 and Myt1l plasmid could be transfected to the fibroblast using commercially available liposomal vehicle such as lipofectamine 2000 (Life Technologies), in this study, a polymeric vehicle was used.

Poly(CBA-ABOL) synthesis and polyplex formation.

Poly(CBA-ABOL) was synthesized by Michael polyaddition of 3.67 g N,N-cystaminebisacrylamide (CBA) (Polysciences, Warrington, PA) and 1.26 g 4-amino-1-butanol (ABOL) (Sigma-Aldrich, St Louis, MO) as described by Lin et al., (*Bioconjug Chem* 18: 138–145 (2007)) and Adler A and Grigsby C et al., (*Molecular Therapy Nucleic Acids* 1: e32; doi:10.1038/mtna.2012.25 (2012)). The reaction product was purified by dialysis (3.5 kDa cutoff) in acidic deionized water (pH 4) and then lyophilized. The polymer was collected in its HCl-salt form (1.63 g, 33% yield). p(CBA-ABOL)/DNA nanocomplexes (polyplexes) were synthesized at a

polymer:DNA mass ratio of 45:1, based on previous studies. Polyplexes were prepared by adding a HEPES buffer solution (20 mmol/l HEPES, 5 wt % glucose, pH 7.4) of p(CBA-ABOL) (900 µg/ml) to a HEPES buffer solution (20 mmol/l HEPES, 5 wt % glucose, pH 7.4) of plasmid DNA (75 µg/ml), followed immediately by vortexing for 20 seconds.

5 Cell culture and transfection.

A total of 40,000 cells (80,000 cells) passage 6-8 PMEF-HLs (Millipore) were seeded per well in 24-well TCPS plates (BD, Franklin Lakes, NJ) at 37 °C and 5% CO₂ in complete PMEF medium: Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose (GIBCO 11960-044) (Invitrogen), 10% FBS (Atlanta Biologicals), 25 µg ml⁻¹ gentamicin (Invitrogen), and 1× GlutaMAX, nonessential amino acids, sodium pyruvate, and β-mercaptoethanol (Invitrogen). In most experiments, poly-D-lysine/laminin-coated PDMS with 2 µm gratings, hierarchical gratings, or unpatterned PDMS were tested; as control samples glass coverslips, or bottom of TCPS wells were tested. Twenty-four hours after seeding, PMEFs were transfected with pmax-BAM or pUNO-AM/pmax-B plasmid cocktails for induced neuronal transdifferentiation. BAM factor plasmids were delivered at an equimolar ratio in all cases. A 2:1 ratio of Lipofectamine 2000 (Invitrogen) volume (µl) to DNA mass (µg) was used for flow cytometry experiments. All transfections were carried out in serum- and antibiotic-free OptiMEM (Invitrogen). OptiMEM was replaced with complete PMEF medium 4 hours after the onset of transfection. Serial transfections for neuronal transdifferentiation were performed in a similar protocol at day 3 and day 5. Forty-eight hours after the final transfection (which was at day 5) was completed, PMEF medium was replaced with N3 neural induction medium containing: DMEM/F-12 (Invitrogen), 25 µg ml⁻¹ bovine insulin (Gemini Bio-Products, West Sacramento, CA), 50 µg ml⁻¹ human apo-transferrin, 30 nmol/l sodium selenite, 20 nmol/l progesterone, 100 µmol/l putrescine (Sigma-Aldrich), 10 ng ml⁻¹ human bFGF2 (Stemgent, Cambridge, MA), and 25 µg ml⁻¹ gentamicin (Invitrogen).

25 Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 min and subsequently blocked with 10% goat serum (Life Technologies) or donkey serum (Merck Millipore, Germany) and permeabilized with 0.1% Triton-X for 60 min. Primary antibodies were diluted in 1% goat or donkey serum and incubated overnight at 4°C followed by overnight incubation with the appropriate secondary antibodies at 4°C. The secondary antibodies used were goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 546, goat anti-rabbit Alexa Fluor 546 (Life Technologies), donkey anti-goat FITC or goat anti-rat FITC (Abcam, England, UK) at the

concentration of 1:500. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 min before mounting with Prolong Gold anti-fade mounting media (Life Technologies). The images of the cells were taken with inverted fluorescence DMIRM microscope (Leica, Germany) or confocal microscope (Leica SP5, Germany).

5 **Table 2.** List of antibodies for staining

Antigen	Dilution	Source	Species
Aldehyde dehydrogenase 1 family, member 1A (ALDH1a1)	1:500	Abcam	Rabbit
β -III-tubulin (TUJ1)	1:350	Sigma Aldrich	Rabbit
β -III-tubulin (TUJ1)	1:500	Abcam	Mouse
Glial fibrillary acidic protein (GFAP)	1:750	Millipore	Mouse
LIM homeobox transcription factor 1, alpha (LMX1a)	1:500	Santa Cruz	Goat
Microtubule associated protein 2 (MAP2)	1:500	Abcam	Mouse
Nestin	1:1000	Abcam	Mouse
Nuclear receptor related 1 protein (NURR1)	1:500	Abcam	Rabbit
Pituitary homeobox 3 (PITX3)	1:1000	Abcam	Rabbit
Sodium channel voltage gated, type 1, alpha subunit (SCN1a)	1:500	Abcam	Rabbit
Tyrosine hydroxylase (TH)	1:500	Pel-Freez	Rabbit
Vesicular transporter of monoamines (VMAT2)	1:250	Santa Cruz	Goat
SOX2	1:250	Santa Cruz	Goat
Doublecortin (DCX)	1:500	Santa Cruz	Goat

Analysis of gene expression

10 Total RNA was extracted from the cells using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Germany). The RNA quantity and concentration were determined using Nanodrop ND-1000. cDNA synthesis was performed with 100 ng of total RNA using SuperScript VILO cDNA Synthesis Kit (Life Technologies) and the reverse transcription was carried out at 42°C for 90 min. Real-time quantitative PCR was carried out using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). TaqMan probes (Applied Biosystems) were used to analyze the following genes of interest: *TH* (Assay ID: Mm00447557_m), *Pitx3* (assay ID:

Mm01194166_g1) and *GAPDH* (assay ID: Mm99999915_g1). Relative quantification was calculated using the $\Delta\Delta C_t$ method which was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene.

Measurement of neuron elongation and alignment

- 5 The elongation and alignment of the neurons were calculated from the TUJ1+ population. The elongation parameter E is calculated as the ratio of the long axis over the short axis on an approximated ellipse [Wong S, et al., *Biomech Model Mechanobiol* 13: 27-39 (2013)] by using ImageJ (NIH). The percentage of aligned cells was obtained by looking at the number of cells that had $E > 3$ and angle of the long axis and the grating < 15 . For each sample, over 200 cells
10 were measured.

Scanning electron microscopy (SEM) of the substrate replicas

- Scanning electron microscopy (SEM) of the replicas was carried out at various magnifications in high vacuum after sputter-coating the samples with platinum at 30 mA for 30 sec. The protein-coated substrates were firstly fixed with 2% gluteraldehyde in 0.1 M sodium cacodylate and 3 mM
15 CaCl_2 , followed by serial dehydration in ethanol and exposure to increasing concentrations of hexamethyldisilazane (HMDS). Lastly, the substrates were dried overnight before SEM imaging.

Statistical analysis

- Data was presented as the mean and standard error of mean where appropriate. All experiments were carried out in duplicates or more. Data analysis was performed by one-way
20 analysis of variance (ANOVA) followed by post-hoc Dunnett's test for multiple comparisons of at least three groups or unpaired Student's t-test for two groups using Prism 6.0 (Graphpad Software Inc.). p values of < 0.05 were considered statistically significant.

RESULTS

Characterization of PDMS and TCPS replicas

- 25 Fidelity of replication with and without extracellular matrix coating was checked using a scanning electron microscope (SEM). The $2 \times 2 \times 2 \mu\text{m}$, $250 \times 250 \times 250 \text{ nm}$, and hierarchical grating patterns of PDMS or TCPS were replicated with good fidelity (Figure 5 and Figure 6). Unpatterned controls replicated from PDMS and TCPS were also examined using SEM to confirm the absence of topographical features.

Effect of MARC topographical cues on differentiation of neural progenitor cells

In order to identify the topographies that would lead to increased midbrain dopaminergic neuron yield, mNPCs were allowed to differentiate into neurons on PDMS replicas of 18-pattern MARC for 19 days. After this 19 day period screening was conducted to identify the percentage of
 5 TUJ1+ (β -III-tubulin) (immature neurons) and GFAP+ (glial fibrillary acidic protein) (astrocytes) cells (Figure 7A). The 18-pattern MARC chip yielded three topographies with significantly higher numbers of TUJ1+ cells compared to the unpatterned control. These were the 2 μ m gratings with 2 μ m spacing and 2 μ m height (2 $\times$ 2 $\times$ 2 μ m gratings or Grating 1), the 250 nm gratings with 250 nm spacing and 250 nm height (250 $\times$ 250 $\times$ 250 nm gratings or Grating 5, and the 2 $\times$ 2 $\times$ 2 μ m
 10 gratings with hierarchical perpendicular 250 $\times$ 250 $\times$ 250 nm grating in the groove (2 $\times$ 2 $\times$ 2 μ m gratings $\perp$ 250 $\times$ 250 $\times$ 250 nm lines or Hierarchical 6). These gratings yielded 47.6 ± 7.0 % ($p < 0.05$), 53.0 ± 2.0 % ($p < 0.01$) and 45.1 ± 12.5 % ($p < 0.05$) of total cells, respectively.

Two topographies generated high neuron-to-astrocyte (N:A) ratios as compared to the unpatterned control. These were 250 $\times$ 250 $\times$ 250 nm gratings (Grating 5) and Hierarchical 6 which
 15 produced N:A ratios of 1.9 ± 0.3 ($p < 0.05$) and 2.5 ± 0.9 ($p < 0.01$), respectively, compared to an N:A of 0.4 ± 0.1 on the unpatterned control. On the other hand, the 2 $\times$ 2 $\times$ 2 μ m gratings with hierarchical parallel 250 $\times$ 250 $\times$ 250 nm grating in the groove (2 $\times$ 2 $\times$ 2 μ m gratings $\parallel$ 250 $\times$ 250 $\times$ 250 nm lines or Hierarchical 7) produced significantly higher GFAP+ cells than TUJ1+ cells ($p < 0.01$).

20 Next, we performed another screen to analyze the percentage of tyrosine hydroxylase (TH+) (dopaminergic-committed neuron) cells after 19 days of differentiation (Figure 7B and Table 3). Five topographical patterns showed significantly higher numbers of TH expressing cells than unpatterned control. These were 2 $\times$ 2 $\times$ 2 μ m gratings (Grating 1) (26.3 ± 3.6 %, $p < 0.001$), 250 $\times$ 250 $\times$ 250 nm gratings (Grating 5) (25.9 ± 3.8 %, $p < 0.001$), Hierarchical 6 (34.1 ± 5.2 %, $p < 0.001$), Hierarchical 7 (24.1 ± 0.5 %, $p < 0.01$), and Microlens 16, which is a convex microlens
 25 of 1.8 μ m diameter with 2 μ m pitch (18.9 ± 3.0 %, $p < 0.05$). To examine TH+ expression in the TUJ1+ immature neuronal sub-population, TH+/TUJ1+ cells were quantified (Figure 7C). Among the topographies, only Microlens 15, which has a 1 μ m pitch concave microlens, produced a lower proportion of TH+ expressing cells than the unpatterned control ($p < 0.05$). No significant
 30 differences in the proportion of TH+ expressing cells among the TUJ1+ population was observed on other topographies compared to the unpatterned control.

Table 3: Comparison between topographical patterns which have significant differences in percentage of TH+ cells.

Compared Patterns	Significance	P-value
Unpatterned vs 1	**	<0.01
Unpatterned vs 5	**	<0.01
Unpatterned vs 6	****	<0.0001
Unpatterned vs 7	**	<0.01
1 vs 9	*	<0.05
1 vs 10	*	<0.05
1 vs 13	***	<0.001
1 vs 15	***	<0.001
2 vs 6	**	<0.01
3 vs 6	**	<0.01
4 vs 6	*	<0.05
5 vs 9	*	<0.05
5 vs 10	*	<0.05
5 vs 13	**	<0.01
5 vs 15	***	<0.001
6 vs 8	**	<0.01
6 vs 9	****	<0.0001
6 vs 10	****	<0.0001
6 vs 11	***	<0.001
6 vs 12	**	<0.01

Compared Patterns	Significance	P-value
6 vs 13	****	<0.0001
6 vs 14	***	<0.001
6 vs 15	****	<0.0001
6 vs 16	*	<0.05
6 vs 17	*	<0.05
6 vs 18	*	<0.05
7 vs 13	**	<0.01
7 vs 15	**	<0.01

Analysis of differentiation of neural progenitor cells on single pattern PDMS substrates

From the results of the 18-pattern MARC chip, we selected and focused on two single-imprinted patterns, 2×2×2 μm gratings and 250×250×250 nm gratings, as well as the double-imprinted pattern, Hierarchical 6, for closer examination of mNPCs differentiation.

We firstly performed quantitative PCR to analyze the gene expression of *TH* and *Pitx3* on the patterned substrates after 11 days and 19 days of differentiation, respectively (Figure 8). The mNPCs differentiated on both grating patterns with the hierarchical grating being associated with a higher expression of *TH* and *Pitx3* than unpatterned substrates. Among the three patterns, the cells that differentiated on Hierarchical 6 had the highest expression of *TH*, while those that had differentiated on 2×2×2 μm gratings had the highest expression of *Pitx3*.

The presence of proteins involved in the maturation and function of dopaminergic neurons were further evaluated by immunofluorescence staining (Figure 9). Neurons were stained with nuclear receptor related protein 1 (NURR1), sodium channel SCN1a, vesicular monoamine transporter 2 (VMAT2), aldehyde dehydrogenase 1 family member A1 (ALDH1a1), and pituitary homeobox 3 (PITX3). The presence of NURR1 indicated that the neurons generated were likely to be midbrain dopaminergic neurons (Figure 9). These neurons also possessed the sodium channels SCN1a and VMAT2, which are membrane proteins that transport dopamine into synaptic vesicles (Figure 9). These markers were robustly expressed in both the single-imprinted (2×2×2

μm gratings and $250\times 250\times 250$ nm gratings) and the double-imprinted (Hierarchical 6) substrates.

Image analyses performed on the single-imprinted patterned substrates showed that the percentage of TH+ (dopaminergic-committed) cells were significantly higher on $2\times 2\times 2$ μm gratings (25.0 ± 0.3 % of total cells; $p < 0.05$) compared to the unpatterned control (Figure 10A). This result is consistent with the data from the MARC chip. After 11 days of differentiation, the percentage of microtubule-associated protein 2 (MAP2+, marker for mature neurons) cells was highest on $2\times 2\times 2$ μm gratings (23.3 ± 2.0 %; $p < 0.001$) amongst the three substrates (Figure 10B). $250\times 250\times 250$ nm gratings also produced significantly more MAP2+ cells (20.4 ± 1.3 %) than the unpatterned control (10.1 ± 1.1 %; $p < 0.01$).

Differentiation on both grating substrates produced more cells that expressed ALDH1a1 (a marker for dopaminergic-committed population) than the unpatterned control, with 40.6 ± 1.1 % ($p < 0.01$) on $2\times 2\times 2$ μm gratings and 30.6 ± 4.0 % ($p < 0.05$) on $250\times 250\times 250$ nm gratings (Figure 10C). Compared to the unpatterned control, we also observed more cells expressing the LIM homeobox transcription factor 1 alpha (LMX1a, a marker for dopaminergic-committed population) on $2\times 2\times 2$ μm gratings (37.3 ± 2.7 %; $p < 0.001$) and $250\times 250\times 250$ nm gratings (26.3 ± 2.2 %; $p < 0.001$) (Figure 10D). $2\times 2\times 2$ μm gratings also produced significantly more pituitary homeobox 3 (PITX3+, a marker for substantia nigra dopaminergic neurons) cells (18.3 ± 1.4 %; $p < 0.01$) than the unpatterned control (Figure 10D). All TH+ cells were positive for ALDH1a1 and LMX1a, and about half of TH+ cells expressed PITX3.

Analysis of elongation and alignment of neurons on patterned PDMS substrates

Longer neurites are correlated with the maturation of the differentiated cells, and thus neuron elongation was quantified using the E value. Compared to the unpatterned control, the neurons were significantly more elongated when differentiated on $2\times 2\times 2$ μm gratings and Hierarchical 6 with an E value of 10.4 ± 0.8 ($p < 0.0001$) and 8.6 ± 0.7 ($p < 0.0001$), respectively (Figure 11A and Figure 11B). In addition, neurite alignment also influences axon guidance towards successful synaptic circuitry. Therefore, the alignment of neurons was compared between the grating patterns. We found that the alignment on $2\times 2\times 2$ μm gratings and Hierarchical 6 were more extensive than $250\times 250\times 250$ nm gratings, with 65.9 % and 93.1 % of the cells aligned along the grating axis, respectively (Figure 11A and Figure 11C). In comparison, only 18.8 % of the cells were aligned along the grating axis on $250\times 250\times 250$ nm gratings.

Analysis of differentiation of neural progenitor cells on TCPS substrates

We also differentiated mNPCs on TCPS substrates with $2 \times 2 \times 2 \mu\text{m}$ gratings and $250 \times 250 \times 250 \text{ nm}$ gratings to determine if the effects of these gratings on the mNPC differentiation apply to different substrate rigidities. Stiffness measurement by atomic force microscopy (AFM) showed the elastic modulus of PDMS (10:1 base/curing ratio) and TCPS to be $3.27 \pm 0.133 \text{ MPa}$ and $2149 \pm 285 \text{ MPa}$ respectively, indicating a considerable difference in stiffness between these two substrates (Figure 12). The percentage of immature neurons and astrocytes (expressing TUJ1 and GFAP, respectively), and the percentage of mature neurons (expressing MAP2) were analyzed after 11 days of differentiation on the TCPS substrates (Figure 13A). $2 \times 2 \times 2 \mu\text{m}$ gratings generated a significantly higher N:A ratio (0.73 ± 0.05 , $p < 0.05$) than the unpatterned control (0.45 ± 0.04) (Figure 13B). A significantly higher percentage of MAP2+ cells was observed on both $2 \times 2 \times 2 \mu\text{m}$ gratings ($45.1 \pm 0.9 \%$; $p < 0.01$) and $250 \times 250 \times 250 \text{ nm}$ gratings ($42.2 \pm 2.0 \%$; $p < 0.05$) compared to the unpatterned control (Figure 13C).

Human iPSC-derived neurons display increased neuronal complexity and branching when grown on a combination of PDMS substrates comprising gratings and pillars

The two stage differentiation method as shown in Figure 4A, using embryoid bodies produced from human iPSCs, produced expanded neural progenitor cells when cultured on substrates with gratings in stage 1 (Figure 4B) and terminally differentiated midbrain dopaminergic neurons on substrates with pillars in stage 2 (Figure 4C). Figure 4B shows bright field images of embryoid bodies in microwells and on patterned substrates at stage 1. Cells were observed to be aligned on the grating axis (arrow) at stage 1. Figure 4C shows immunofluorescence images of cells expressing tyrosine hydroxylase (TH), a dopaminergic neuron marker at stage 2 on patterned substrates. In particular, when the cells are grown on substrates with pillars in stage 2, they display a highly differentiated morphology with significant neurite branching.

Immunostaining for dopaminergic neuronal markers (TUJ1, MAP2, PITX3, LMX1a and FOXA2) on human induced pluripotent stem cell (iPSC)-derived cells cultured on unpatterned, gratings, and pillars PDMS substrates in stage 2 showed robust expression when on patterned substrates (Figure 14).

Quantification and data analysis of TUJ1-positive (neuronal) cells after 21 day differentiation on unpatterned, gratings and pillars PDMS substrates in stage 2 showed that both gratings and pillars produced higher percentages of TUJ1-positive cells than unpatterned (Figure 15A).

Quantification and data analysis of TH-positive (dopaminergic) cells after 21 day differentiation on unpatterned, gratings and pillars PDMS substrates in stage 2 showed differences in percentage TH+ cells (Figure 15B) which was more pronounced when the data was normalised to the unpatterned substrates; significant with respect to gratings (Figure 15C). Both gratings and pillars in stage 2 produced an increased ratio of TH+:TUJ1+ cells over unpatterned (Figure 15D). Quantification and data analysis of FOXA2-positive (midbrain DA) cells on unpatterned, gratings, and pillars PDMS substrates in stage 2 showed that substrates with gratings and pillars had significantly more cells expressing FOXA2 than unpatterned substrates (Figure 15E).

Morphology and synaptic activity of TH+ neurons is more mature on stage 2 pillars substrates after 21 day differentiation.

Figure 16A shows representative morphology of cells on the three substrates, whereby cells on gratings were aligned on the gratings axis. The average neurite length per neuron was longer for cells grown on gratings in stage 2 than for those grown on pillars substrates (Figure 16B), whereas the number of branch points (Figure 16C), neuronal complexity (Figure 16D) and number of terminals per neuron (Figure 16E), were significantly greater for neurons grown on pillars substrates compared to gratings ($p < 0.01$). In addition, the percentage of cells with a spontaneous postsynaptic current was higher in TH +ve neurons differentiated on pillars (Figure 16F) and can be detected as early as 4 weeks post differentiation (Figure 16G; arrowhead).

Repetitive action potential capability of TH+ neurons is more pronounced on stage 2 pillars substrates after 5 weeks post differentiation.

Figure 17 shows representative tracings of firing patterns on gratings at 4 weeks and 5 weeks post differentiation. (A) Cells were capable of firing a repetitive action potential as a response to current injection as early as 4 weeks post differentiation. Spontaneous synaptic activity (arrowhead) was recorded as early as 4 weeks post differentiation and is more frequently observed at 5 weeks. (B) The percentage of differentiated neurons capable of repetitive firing at 5 weeks post differentiation was greater for cells on pillars than cells grown in stage 2 on unpatterned substrates or on gratings. Five to nine neurons were recorded from three experiments per condition.

Parkinson's disease patient-derived iPSCs can be successfully differentiated to midbrain dopaminergic neurons on patterned substrates.

Figure 18 shows dopaminergic neuronal markers (Tuj1, TH and LMX1a) are expressed on human iPSC-derived cells from a Parkinson's disease patient with a LRRK2 mutation when grown on gratings, and pillars PDMS substrates. The robust expression indicates that patient-derived iPS cells can be successfully differentiated to midbrain dopaminergic neurons on patterned substrates.

Figure 19 (A) shows representative tracings of firing patterns on gratings at 4 weeks and 5 weeks post differentiation. Neurons were capable of firing a repetitive action potential as a response to current injection as early as 4 weeks post differentiation. (B) The data for the percentage of differentiated cells capable of repetitive firing at 5 weeks post differentiation showed that more neurons differentiated on gratings and pillars were capable of repetitive firing than on unpatterned substrates.

Non-viral direct transdifferentiation of somatic cells to neuronal cells

As previously described in Adler A and Grigsby C et al., (*Molecular Therapy Nucleic Acids* 1: e32; doi:10.1038/mtna.2012.25 (2012)), somatic cells such as fibroblasts can be transdifferentiated by using plasmids encoding neuronal transcription factors (Brn2, Ascl1, Myt1l) with a carrier such as bio reducible linear poly(amido amine). With repeated dosing, transdifferentiation can be achieved at low toxicity.

As reported in Adler et al., (*Molecular Therapy Nucleic Acids*, 2012), serial 0.5µg /cm² doses of reprogramming factors delivered at 48-hour intervals (at day 1, day 3 and day 5) produced up to 7.6% Tuj1+ cells after culturing in N3 medium for 10 days. While an increase of Tuj1+ cells were observed on the 2 µm gratings (to 9%), a significant increase of Tuj1+ cells (to 14%) can be derived on the hierarchical platform shown in Figure 5.

DISCUSSION

In our studies, we have demonstrated the importance of topographical cues in promoting neuronal differentiation by adopting three different differentiation methods to derive neurons. Importantly, a 2 stage differentiation involving different topographical cues may lead to greater morphological and functional differentiation of dopaminergic neurons.

Based on a systematic screening of the MARC chip under the same differentiation conditions, three topographies were first identified that improved the yield of dopaminergic-committed cells from murine neuronal progenitor cells (mNPCs). These were $2\times 2\times 2\text{ }\mu\text{m}$ gratings, $250\times 250\times 250\text{ nm}$ gratings, and Hierarchical 6. Between the two single-imprinted grating patterns, the micron-width $2\times 2\times 2\text{ }\mu\text{m}$ gratings produced greater yields of subtype-specific and region-specific dopaminergic neurons, as well as longer neurites aligned along the grating axis. Based on this analysis, we found that $2\times 2\times 2\text{ }\mu\text{m}$ gratings is the most effective topography at inducing differentiation of mNPCs to dopaminergic neurons in synergy with the appropriate biochemical cues. The double-imprinted grating pattern, Hierarchical 6, also performed efficiently and was also comparable to $2\times 2\times 2\text{ }\mu\text{m}$ gratings.

Substrate topographies were previously demonstrated to affect neuronal specification and differentiation propensity during neural stem/progenitor cell differentiation. [Moe AAK, et al., *Small* 8: 3050-3061 (2012); Yang K, et al., *ACS Applied Materials & Interfaces* 5: 10529-10540 (2013)]. Most studies however have focused on comparing single topographical patterns with the same dimension and shape. Considering different topographical patterns play different roles in neural stem cell differentiation, we proposed the importance of identifying specific patterns to improve derivation efficiency of subtype specific neurons. In line with this, the differentiation of mNPC to dopaminergic neuron subtype (TH+, ALDH1a1+, PITX3+, LMX1a+) was examined on a variety of patterned structures on the MARC chip. In comparison to a related study by Moe et al. [Moe AAK, et al., *Small* 8: 3050-3061 (2012)], the media composition and the protocol used here for deriving subtype-specific neurons may follow a different neural induction pathway, which is further guided by topographical cues. This led to a different proportion of neuronal cells (TUJ1+ cells) but a consistent yield of astrocytes (GFAP+ cells) on the different patterns, thus reflecting different outcomes in these studies.

The aspect ratio of the gratings was shown to be an important determinant of mesenchymal stem cell elongation and alignment [Wong S, et al., *Biomech Model Mechanobiol* 13: 27-39 (2013); Chua J, et al., *Biomaterials* 35(27): 7750-61 (2014)]. Therefore, the spacing and height of the gratings play important roles in the topographical construct that determines cell behaviour and cell fate. By comparing grating topographies on the MARC chip, we found that $2\times 2\times 2\text{ }\mu\text{m}$ gratings and $250\times 250\times 250\text{ nm}$ gratings gave the highest yield of TUJ1+ and TH+ cells, which are attributed to their grating aspect ratio of 1 (grating width : spacing : height of 1:1:1). By decreasing this aspect ratio (Gratings 2-4) (Figures 7A and 7B) the extent of neural induction,

and dopaminergic subtype specification, was reduced, suggesting that the aspect ratio of these patterns is critical in ensuring optimal lineage guidance.

From the MARC chip screening, we identified that Hierarchical 6 yielded significantly more TUJ1+ and TH+ cells, but yielded the least number of astrocytes, compared to the unpatterned control. We focused on single-imprinted patterns (2×2×2 μm gratings and 250×250×250 nm gratings) for single-pattern analysis due to their practical applicability for scaled-up fabrication and manufacturing. However, double-imprinted patterns such as the hierarchical gratings also provided similar enhancement, albeit via potentially distinct mechanisms. Indeed, Hierarchical 6, which is a combination of two grating patterns, showed higher gene expression of *TH* and *Pitx3* and fewer astrocytes than the unpatterned control, suggesting that double-imprinted patterns also provide a significant advantage in the derivation of midbrain dopaminergic neurons. Most methods of DA neuron derivation can require up to 80 days of cell culture and rely heavily on the constant input of expensive biochemical components. The hierarchical gratings can produce a purer population of neurons four times faster and through the reduction of cell culture duration can cut production cost drastically.

Compared to the unpatterned control, there was no significant difference in the proportion of cells showing dopaminergic neuron commitment (TH+ cells) among the population of TUJ1+ cells, and this was regardless of the substrate topography presented. One exception to this was Microlens 15, which seems to have a negative effect on the dopaminergic subtype specification (Figure 7C). Topographical gratings enhance neuronal differentiation and indeed, an increase in the neuronal population was still observed. This indicates a synergistic effect between chemical induction and topographical cues, and this may explain the increase in TH-expressing cells on the topography.

Key midbrain dopamine regulators PITX3 and LMX1a, which play important roles during neurogenesis and neurotransmitter phenotype determination of dopaminergic neurons [Hong S, et al., *Stem Cells Dev* 23: 477-487 (2014); Cai J, et al., *Stem Cells* 27: 220-229 (2009); Nunes I, et al., *Proc Natl Acad Sci USA* 100: 4245-4250 (2003)], were highly expressed in cells differentiated on 2×2×2 μm gratings. Transduction of NURR1 and PITX3 was shown to promote embryonic stem cell differentiation into a dopaminergic phenotype [Martinat C, et al., *Proc Natl Acad Sci USA* 103: 2874-2879 (2006)]. Importantly, the expression of ALDH1a1, which is the transcriptional target of PITX3, was also increased on this topography [Jacobs F, et al., *Development* 134: 2673-2684 (2007)]. As ALDH1a1 is expressed specifically in dopaminergic

neurons in the ventral midbrain and is decreased in Parkinson's disease patients [Galter D, et al., *Neurobiol Dis* 14: 637-647 (2003); Anderson D, et al., *Brain Res* 1408: 81-87 (2011)], these results suggest that the dopaminergic phenotype derived here is physiologically relevant as cellular models and can be favourably specified.

- 5 Late stage markers of dopaminergic neurons including NURR1, voltage gated sodium channels (SCN1a), and vesicular monoamine transporter 2 (VMAT2) were also expressed in the neurons. This further supports the successful midbrain dopaminergic neuron subtype specification on the topographies. Sodium channel development suggests that the neurons possess the components required for the successful firing of action potentials [Caputi L, et al., *Synapse* 48: 123-130
10 (2003)]. Furthermore, 2×2×2 µm gratings produced the highest percentage of MAP2+ cells, as well as dopaminergic precursor markers, which indicate greater differentiation potential into mature neurons with functional components.

Substrate stiffness has been shown to affect differentiation of stem cells towards specific lineages. Engler *et al.* showed that mesenchymal stem cells were neurogenic when
15 differentiated on substrates of comparable stiffness to the brain tissue (~700 Pa), but myogenic and osteogenic on stiffer substrates [Engler A, et al., *Cell* 126: 677-689 (2006)]. Similarly, neural stem cell differentiation was also affected by the substrate stiffness, where softer substrates favour neural differentiation and stiffer substrates favour oligodendrocyte differentiation [Leipzig N, Shoichet M. *Biomaterials* 30: 6867-6878 (2009)]. Here, we showed that despite differentiation
20 on stiffer substrates such as TCPS, topography-enhanced differentiation was observed for the mNPCs. The influence of these patterns on neuronal differentiation may thus play a larger role than substrate stiffness effects, which have an inevitable influence to a certain extent and have to be considered along with the chosen pattern.

Other methods to derive midbrain dopaminergic neurons from pluripotent stem cells have
25 obtained higher yields than was observed for our method of mNPC differentiation [Kriks S, et al., *Nature* 480: 547-551 (2011); Perrier A, et al., *Proc Natl Acad Sci USA* 101: 12543-12548 (2004)]. However, these studies involved 80 days of culture. Hence, the lower dopaminergic neuron yield in mNPC differentiation, which was observed after 19 days in our study, may due to a premature time point. In addition, the only ventralising factors used in this study to achieve
30 specification were SHH and FGF8. This is opposed to the usual pluripotent stem cell differentiation protocols, which rely on a high dependence of biochemical input, such as the dual SMAD inhibition pathway. Recent studies have shown that non-mesencephalic neural stem cells

are more restricted in their developmental capacity to generate midbrain dopaminergic neurons, suggesting differences in the epigenetic state of these cells in the responsiveness to dopaminergic induction signals [Studer L. *Prog Brain Res* 200: 243-263 (2012); Rössler R, et al., *Neuroscience* 170: 417-428 (2010); Mohamed A, et al., *J Neurosci Res* 90: 529-539 (2012)].

5 Hence the hippocampal origin of mNPCs may limit their potential in developing into mesencephalic neurons. In particular, the epigenetic differences between the subventricular zone and hippocampal neurons account for differences in miRNA regulation of neurogenesis [Mohamed A, et al., *J Neurosci Res* 90: 529-539 (2012)].

By using human induced pluripotent stem cells (iPSCs), we have also examined the ability of
10 topographical patterns to influence the differentiation of hPSCs into subtype-specific and regionalized dopaminergic (DA) neurons. We have successfully established a step-by-step method to differentiate human pluripotent stem cells into ventral midbrain DA neurons on topographical patterned substrates. We have tested our method on induced pluripotent stem (iPS) cells to show the robustness of the protocol, on normal subjects as well as patient cell
15 lines. Results indicate that these pluripotent stem cells can be differentiated to the midbrain DA neuronal lineage on the optimized two stage differentiation process with the use of topography. Among the different patterned substrates used in the second stage of differentiation (gratings and pillars) that was tested, gratings showed more midbrain DA neurons that were derived from human pluripotent stem cells, suggesting that gratings may be good for initial lineage
20 commitment. For functionality, midbrain DA neurons that were derived on pillars were better, based on the increased neuronal complexity and branching. The electrophysiology studies also indicated that a larger proportion of cells that were differentiated using pillars substrates were more capable of firing repetitive action potentials. Interestingly and notably, adopting this two stage topography protocol for human induced pluripotent stem cells (iPSC)-derived cells from
25 Parkinson's disease with LRRK2 mutation showed robust expression of Beta-III tubulin (TUJ1), tyrosine hydroxylase (TH), LIM homeobox transcription factor 1 alpha (LMX1a), indicating patient derived iPS cells can be successfully differentiated to midbrain DA neurons on patterned substrates (Figure 18). Examining the electrophysiology properties of human induced pluripotent stem cell (iPSC)-derived cells from PD patient with LRRK2 mutation on unpatterned, gratings, and pillars PDMS substrates demonstrated that a higher percentage of neurons on gratings and
30 pillars were capable of repetitive firing than on unpatterned substrate (Figure 19), showing that patient-derived iPSCs can be matured into DA neurons. Therefore, the use of gratings for the initial lineage commitment of midbrain DA neurons, followed by pillars for the maturation of DA

neuron functionality will allow for an accelerated DA differentiation process, the development of robust cell models that will provide novel insights into mechanisms underlying DA neuronal development and ultimately discover new therapeutic approaches for Parkinson's disease.

Besides using induced pluripotent stem cells to generate patient specific dopaminergic neurons [Lu H, et al., *Biomaterials* 35: 2816-2826 (2014); Hartfield EM, et al., *PloS one* 9: e87388 (2014); Soldner F, et al., *Cell* 136: 964-977 (2009)] and also for pluripotent stem cells to engraft into animal models [Kriks S, et al., *Nature* 480: 547-551 (2011); Kirkeby A, et al., *Cell reports* 1: 703-714 (2012)], new methods are being optimized for the direct reprogramming of fibroblasts into neurons to circumvent the long tedious differentiation process. However, efficiencies of reprogramming have been low, with approximately 1.5% - 2.9% of adult human fibroblasts being converted using miRNA [Ambasudhan R, et al., *Cell Stem Cell* 9: 113-118 (2011)]. Other studies have generated dopaminergic neurons directly from mouse or human fibroblasts with efficiency levels of between 1-5% for TH+ cells [Caiazzo M, et al., *Nature* 476: 224-227 (2011)]. Epigenetic regulation was shown to influence cell fate determination and self-renewal, and epigenetic modifications on neural stem cells could also increase neuronal differentiation. However, reprogrammed cells showed greater efficiency in generating region-specific neuronal subtypes [Deleidi M, et al., *PloS one* 6: e19926 (2011)]. As topography influences neuronal subtype derivation, it is speculated that topography also play a role in the epigenetic regulation that determines cell fate decisions. The use of topography in a transient or prolonged manner may well be the next step to improve reprogramming efficiencies, and subsequently, differentiation of stem cells into midbrain dopaminergic neurons.

Several mechanisms have been suggested to facilitate topography-induced enhancement of neuronal differentiation. Mechanosensing of topography through the focal adhesion is essential for topography-induced enhancement in neuronal differentiation [Migliorini E, et al., *Biotechnol Bioeng* 108: 2736-2746 (2011); Teo BKK, et al., *ACS Nano* 7: 4785-4798 (2013); Frey M, et al., *Biophys J* 90: 3774-3782 (2006)], while the focal adhesion kinase (FAK) is critical in mechanosensing [Teo BKK, et al. *ACS Nano* 7: 4785-4798 (2013); Wang H, et al., *Proc Natl Acad Sci USA* 98: 11295-11300 (2001)]. In the case of gratings, the grooves and gaps provide more contact points for the formation of focal adhesions [Yang K, et al., *ACS Applied Materials & Interfaces* 5: 10529-10540 (2013)]. We observed that cells sit within the grating groove of the 2×2×2 µm gratings, which may allow for the enhanced formation of focal adhesion complexes with increased elongation of neurites. Neurons that are more elongated and aligned to the

grating patterns may contribute to the development of successful axon guidance towards successful synaptic circuitry.

Cytoskeletal elongation in cells guided by grating patterns has also been proposed to involve the transfer of tensional force to the nucleus, which influences signal transduction and gene expression, and thus stem cell differentiation [Yim E, et al., *Exp Cell Res* 313: 1820-1829 (2007); Teo BKK, et al., *ACS Nano* 7: 4785-4798 (2013)]. Other possible reasons why 2×2×2 μm gratings produced the highest yield of dopaminergic neurons are that the collection of extracellular matrix and growth factors secreted by the cells within the grooves provide an ideal microenvironment for the mNPCs. The enhanced neuronal differentiation, which was observed with increasing grating depth, has also been suggested to be due to the depth-sensing ability of the neurites. This ability is facilitated by filopodia adhesion and neurite bending, both of which have been closely associated with neurite alignment and growth [Chua J, et al., *Biomaterials* S0142-S9612 (2014)].

CONCLUSION

We have shown that micro- and nano-topographies influence dopaminergic subtype specification and differentiation, and can be potentially used to improve derivation efficiencies of other subtype specific neurons and adapted for other clinical uses. As the role of topography in aiding cell fate determination is demonstrated, we have begun to understand the mechanisms that drive topography induced downstream signalling and determination of cell fate. We have successfully established a step-by-step method to differentiate human pluripotent stem cells into ventral midbrain DA neurons on topographical patterned substrates. We have tested our method on induced pluripotent stem (iPS) cells to show the robustness of the protocol, on normal subjects as well as patient cell lines. Results indicate that these pluripotent stem cells can be differentiated to the midbrain DA neuronal lineage on the optimized protocol with the use of topography. In different patterned substrates that we have tested, gratings showed more midbrain DA neurons that were derived from human pluripotent stem cells, suggesting that gratings may be good for initial stage 1 lineage commitment. For functionality, midbrain DA neurons that were derived on pillars in stage 2 were better, based on the increased neuronal complexity and branching. Electrophysiology studies also showed that more cells that were differentiated on pillars in stage 2 were capable of firing repetitive action potentials. Therefore, we suggest the use of gratings for the initial lineage commitment of midbrain DA neurons, followed by pillars for the last push of functionality. These midbrain DA neurons can be

translated into a wide range of clinical applications, and also provide novel insights into the mechanisms that underlie dopaminergic neuron development *in vivo*, which are important in the discovery of new therapeutic approaches for Parkinson's Disease.

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CLAIMS

1. A method of enhancing the differentiation of cells into neurons, wherein the cells are selected from the group multipotent stem cells, pluripotent stem cells and somatic cells, comprising seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein
5 the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium.
2. The method of claim 1, further comprising the steps;
 - a) maintaining the seeded cells in medium;
 - 10 b) inducing neuronal lineage phenotype by withdrawal of growth factors and/or inhibitors;
 - c) replacing the medium of b) with neural patterning medium.
3. The method of claim 2 comprising two stages;
 - i) seeding the cells onto a substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and
15 hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, and contacting the cells with medium to induce neuronal lineage commitment, and
 - ii) removing the cells from the substrate of stage i) after a period of contact with said medium and re-seeding the removed cells onto a substrate patterned with nano- and/or
20 micro pillars, and contacting the cells with neural patterning medium.
4. The method of claim 2 or 3, wherein the neural patterning medium in c) comprises Neurobasal and DMEM/F12 in 1:1 ratio, 1x B27 supplement, 0.25x N2 supplement, 100ng/ml FGF-8, 200ng/ml Sonic Hedgehog and 1µg/ml laminin or, if the cells are pluripotent stem cells, the medium in c) comprises BDNF (20 ng/ml), GDNF (10 ng/ml) and ascorbic acid (200 µM) for
25 3 days, and then DAPT (2.5 µM) is added to the medium from day 4 of stage (ii).
5. The method of any one of claims 1 to 4, wherein the seeded cells are differentiated into dopaminergic neurons.

6. The method of any one of claims 3 to 5, wherein the seeded cells are contacted with the neural patterning medium for at least 5 to 30 days, preferably for at least 7 to 19 days.
7. The method of any one of claims 1 to 3, wherein the cells are somatic cells and they are trans-differentiated by non-viral transfection of one or more transcription factors.
- 5 8. The method of claim 7, wherein the somatic cells are fibroblasts.
9. The method of any one of claims 1 to 8, wherein the hierarchical secondary structures are selected from the group comprising gratings, pillars, columns and lens.
- 10 10. The method of any one of claims 1 to 9, wherein the dimensions of the gratings are 250 nm to 2 μ m wide with 250 nm to 2 μ m spacing and 250 nm to 2 μ m height, and the hierarchical secondary structures are gratings with dimensions of 100 nm to 250 nm wide with 100 nm to 250 nm spacing and 100 nm to 250 nm height; and the pillars are 0.5 μ m to 5 μ m diameter with 8 μ m to 12 μ m pitch.
11. The method of any one of claims 1 to 10, wherein the gratings are parallel.
12. The method of any one of claims 1 to 11, wherein the hierarchical secondary gratings are perpendicular to the longitudinal axis of the gratings.
- 15 13. The method of any one of claims 1 to 12, wherein the gratings have an aspect ratio of 1 (grating width: spacing: height of 1:1:1).
14. The method of any one of the previous claims, wherein the substrate is coated with extracellular matrix proteins prior to seeding.
- 20 15. The method of claim 14, wherein the extracellular matrix proteins are selected from at least one from the group comprising poly-L-ornithine, fibronectin and laminin.
16. The method of any one of claims 3 to 15, comprising a two stage method of enhancing the differentiation of embryoid body (EB) cells, derived from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), into dopaminergic neurons comprising;
25 i) seeding dissociated embryoid body (EB) cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with parallel grating structures, and contacting the cells with medium comprising SMAD inhibitor,

Noggin, sonic hedgehog (SHH) and CT99021 to induce neuronal lineage commitment; and

ii) removing the cells from the substrate of stage i) after a period of contact with said medium and re-seeding the removed cells onto a substrate coated with laminin, fibronectin and poly-L-ornithine, wherein the substrate has been patterned with pillars or gratings, and contacting the cells with medium comprising BDNF, GDNF and Ascorbic Acid; and

adding DAPT to the medium from day 4.

17. The method of any one of the previous claims, wherein the substrate is selected from the group comprising polymethylsiloxane (PDMS) and tissue culture polystyrene (TCPS).

18. A substrate patterned with nano- and/or micro-grating structures, wherein the nano- and/or micro-grating structures are selected from gratings and hierarchical gratings, wherein said hierarchical gratings comprise said gratings with secondary structures at the base of and within the grooves of said gratings, wherein the dimensions of the gratings are 250nm to 2µm wide with 250nm to 2µm spacing and 250nm to 2µm height.

19. The substrate of claim 18, wherein the hierarchical secondary structures are selected from the group comprising gratings, pillars, columns and lens.

20. The substrate of claim 18, wherein the hierarchical secondary structures are gratings with dimensions of 100nm to 250nm wide with 100nm to 250nm spacing and 100nm to 250nm height.

21. The substrate of claim 18, wherein the gratings are parallel.

22. The substrate of claim 18 or 19, wherein the hierarchical secondary gratings are perpendicular to the longitudinal axis of the gratings.

23. The substrate of any one of claims 18 to 22, wherein the gratings have an aspect ratio of 1 (grating width: spacing: height of 1:1:1).

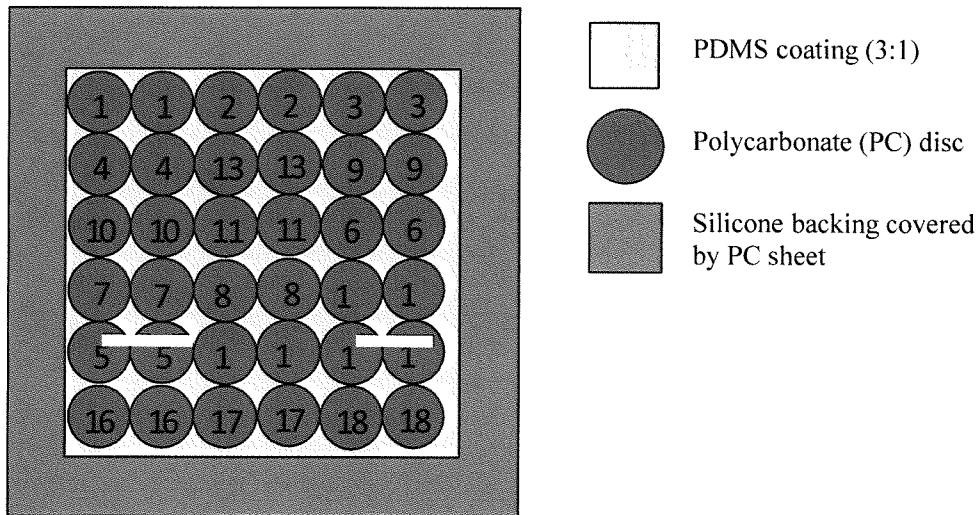
24. The substrate of any one of claims 18 to 23, wherein the substrate is coated with extracellular matrix proteins.

25. The substrate of claim 24, wherein the extracellular matrix proteins are selected from at least one from the group comprising poly-L-ornithine, fibronectin and laminin.
26. The substrate of any one of claims 18 to 25, wherein the substrate is selected from the group comprising polymethylsiloxane (PDMS) and tissue culture polystyrene (TCPS).
- 5 27. Use of dopaminergic neurons produced according to any one of claims 1 to 6 and 9 to 17 for the treatment of Parkinson's disease.
28. A method of treatment of Parkinson's disease, comprising administering to a subject in need thereof an efficacious amount of dopaminergic neurons produced according to any one of claims 1 to 6 and 9 to 17.
- 10 29. The use of dopaminergic neurons produced according to any one of claims 1 to 6 and 9 to 17 to screen for compounds with activity in ameliorating or treating Parkinson's disease, in vitro testing or drug screening related to Parkinson's disease or diseases related to dopaminergic neurons.
30. A method of testing compounds for activity in ameliorating or treating Parkinson's disease,
15 comprising the steps:
- (a) contact dopaminergic neurons obtained according to any one of claims 1 to 6 with the test compound, and
 - (b) quantitate the level of any one or more from the group comprising dopamine production, TH expression, Lmx1a expression, Foxa2 expression PITX3 expression,
20 MAP2 expression, NURR1 expression, ALDH1a1 expression, SCN1a expression, VMAT2 expression, Pax6 expression and neuron complexity; and/or
 - (c) measure the electrophysiological profile of said neurons; and
 - (d) compare the level quantitated in (b) and/or measured in (c) with the level in untreated dopaminergic neurons, wherein a difference in level indicates the compound has
25 Parkinson's disease-ameliorating activity.
31. The method or substrate of any one of the previous claims, wherein the gratings are 2µm wide with 2µm spacing and 2µm height, the hierarchical secondary gratings are 250nm wide with 250nm spacing and 250nm height and are perpendicular to the longitudinal axis of said gratings and the pillars are 2 µm diameter with 12 µm pitch.

FIGURES

FIGURE 1

A



Category	No.	Pattern description
Gratings	1	2 μ m gratings $\times$ 2 μ m spacing $\times$ 2 μ m height
	2	1 μ m gratings $\times$ 2 μ m spacing $\times$ 2 μ m height
	3	2 μ m gratings $\times$ 1 μ m spacing $\times$ 1 μ m height
	4	250nm gratings $\times$ 250nm spacing $\times$ 150nm height
	5	250nm gratings $\times$ 250nm spacing $\times$ 250nm height
Hierarchy gratings	6	2 μ m gratings $\times$ 2 μ m space & perpendicular 250nm grating/250nm spacing
	7	2 μ m gratings $\times$ 2 μ m space & parallel 250nm grating/250nm spacing
	8	2 μ m gratings $\times$ 2 μ m space & 250nm dimple overlay
Pillars	9	2 μ m diameter pillars with 10 μ m pitch
	10	500nm diameter pillars with 10 μ m pitch
	11	130nm diameter pillars with 400nm pitch
	12	250nm diameter pillars with 500nm pitch
Wells	13	1 μ m diameter wells with 8 μ m pitch
Microlens	14	1 μ m pitch microlens (convex)
	15	1 μ m pitch microlens (concave)
	16	1.8 μ m diameter with 2 μ m pitch microlens (convex)
	17	1.8 μ m diameter with 2 μ m pitch microlens (concave)
Motheye	18	Motheye with 270nm pitch

FIGURE 1 cont.

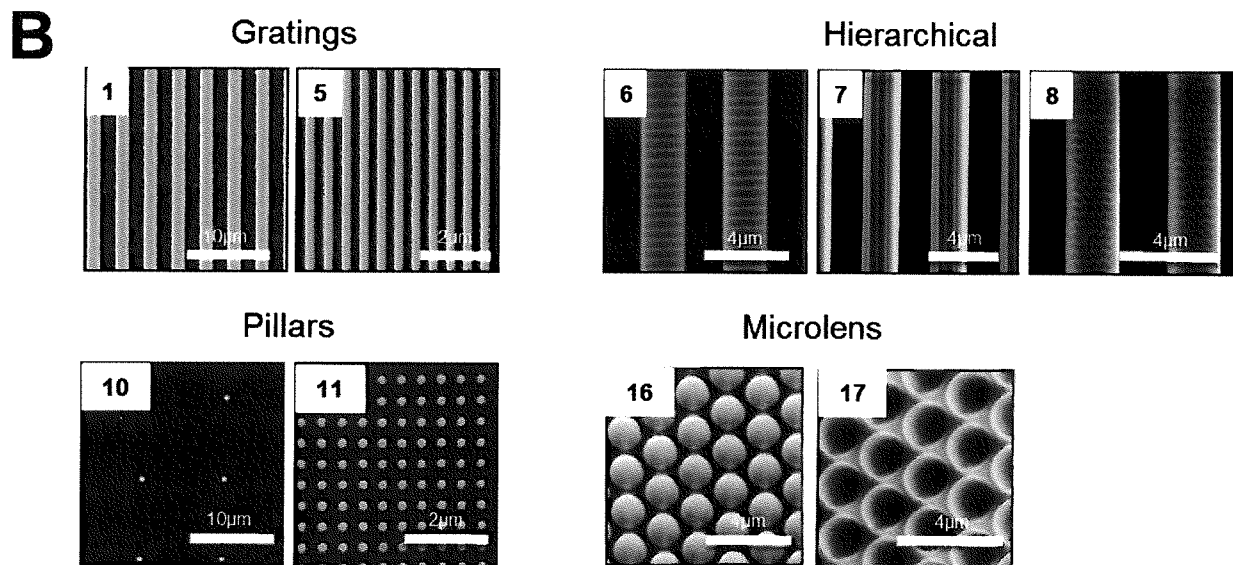


FIGURE 2

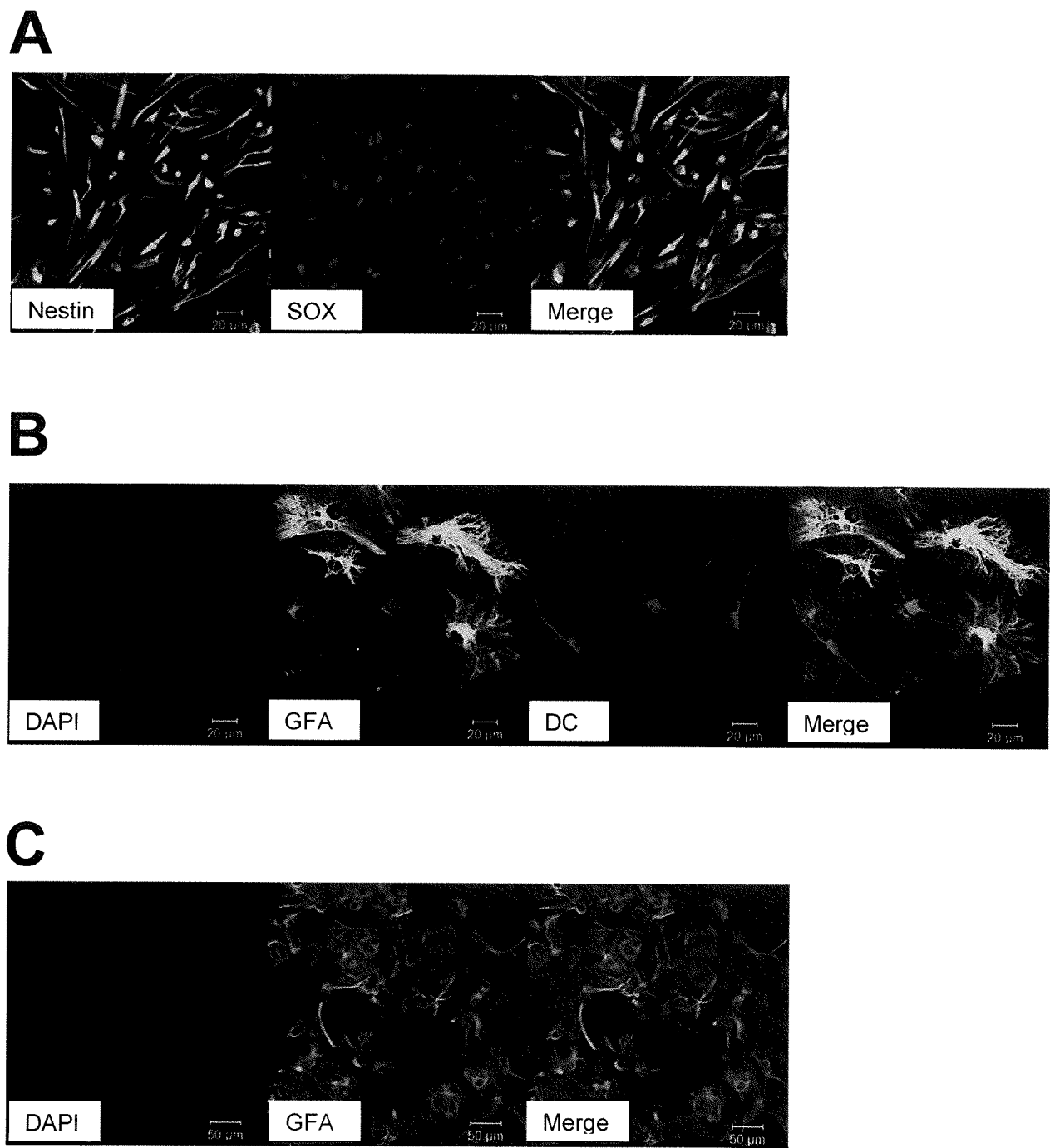


FIGURE 3

A

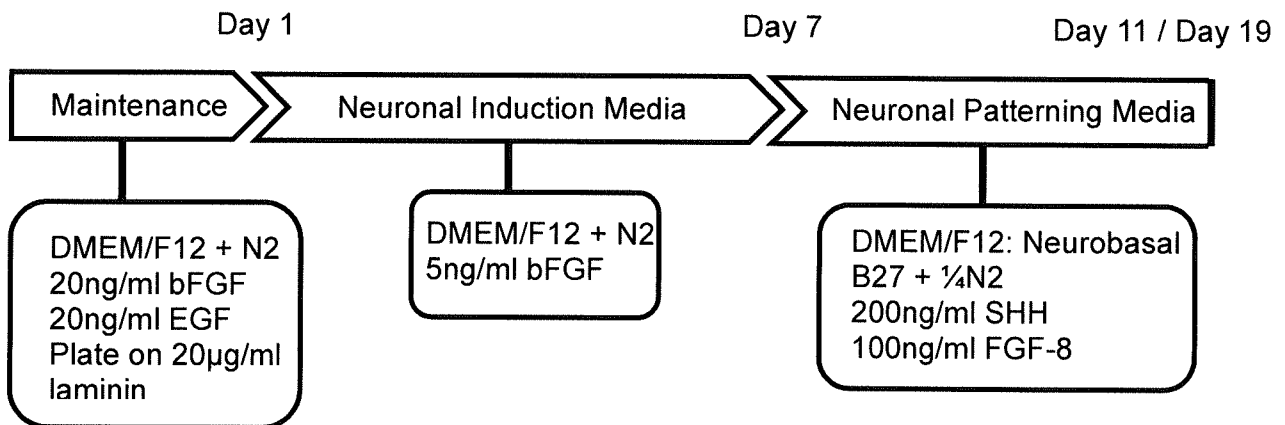


FIGURE 4

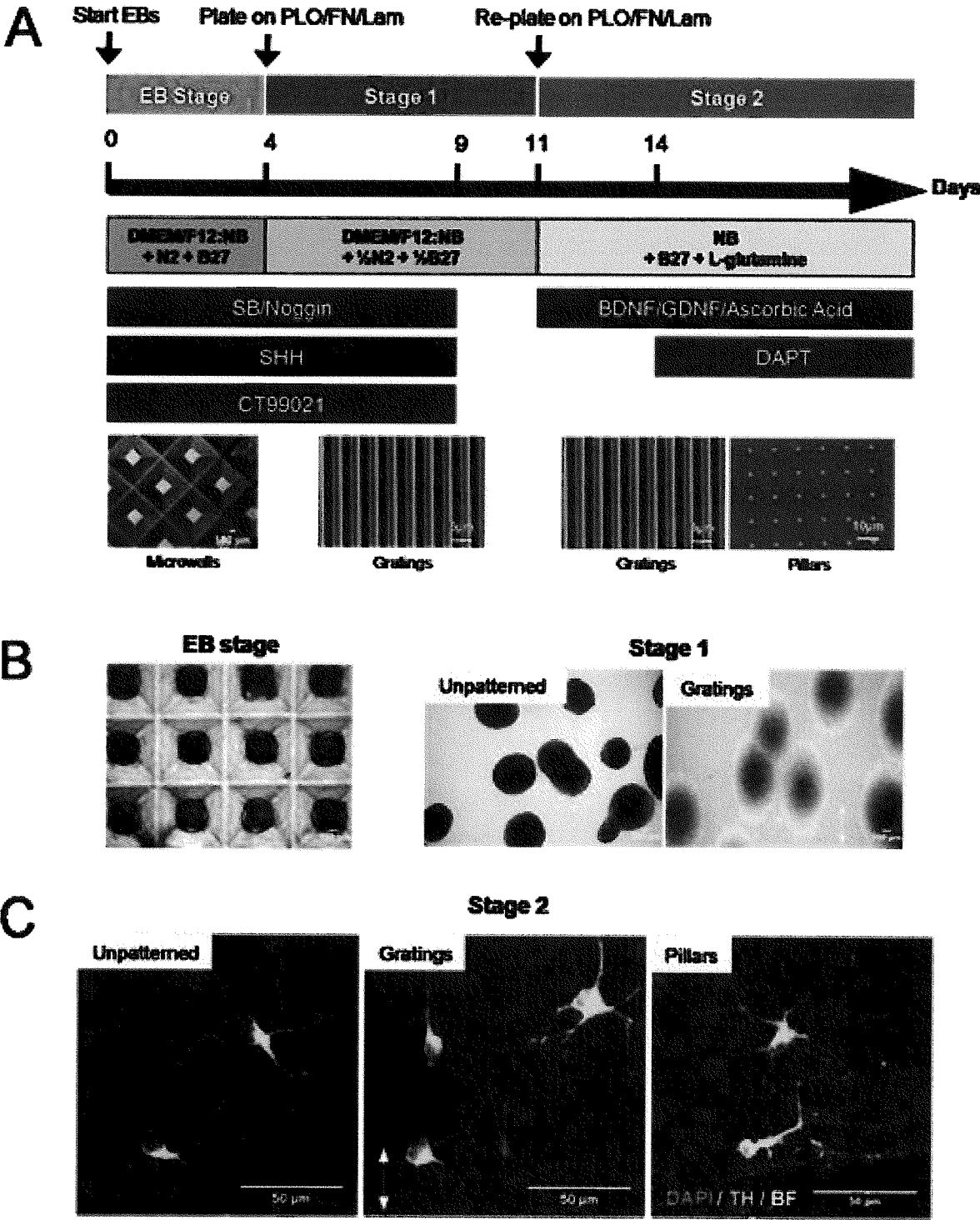


FIGURE 5

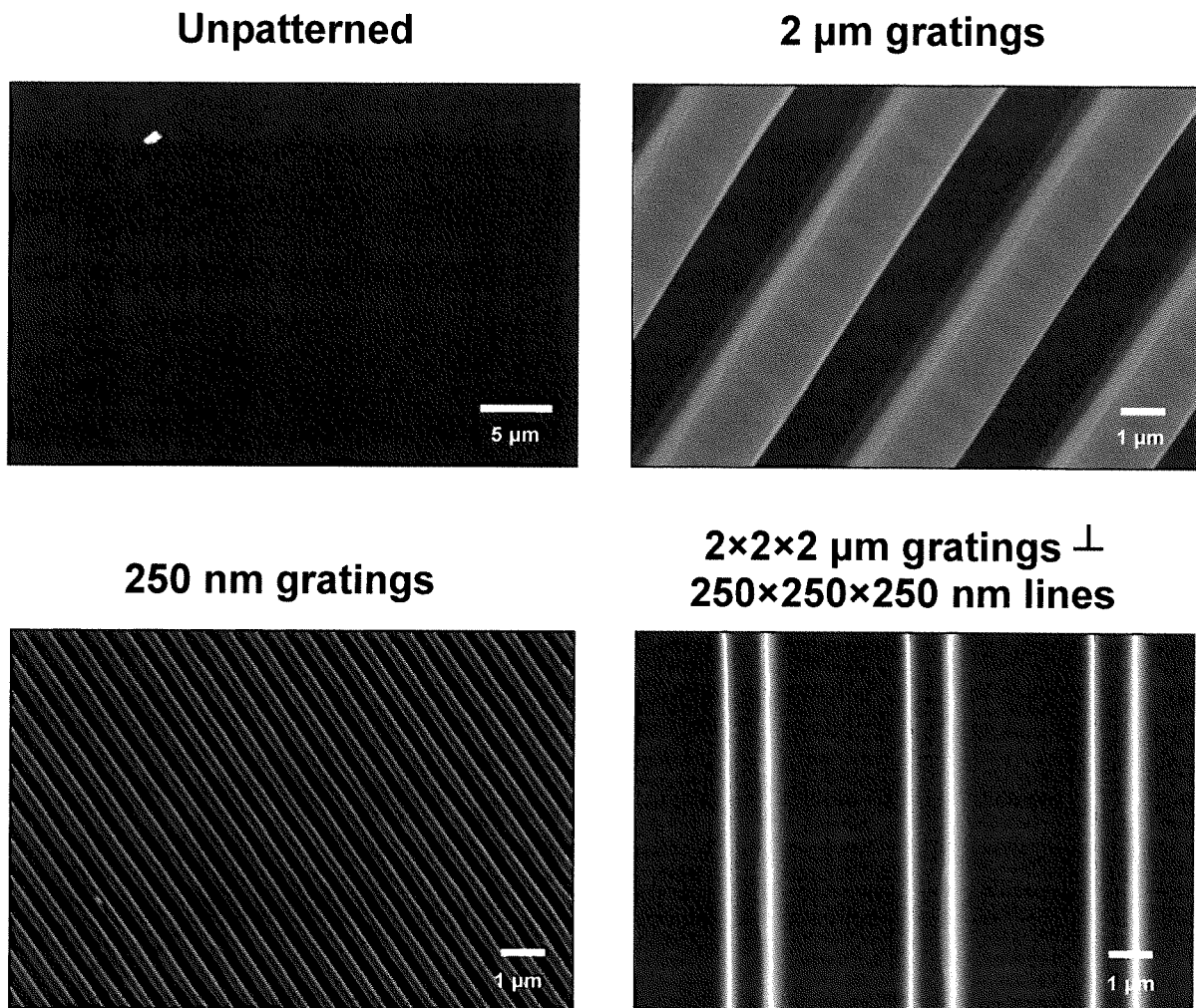
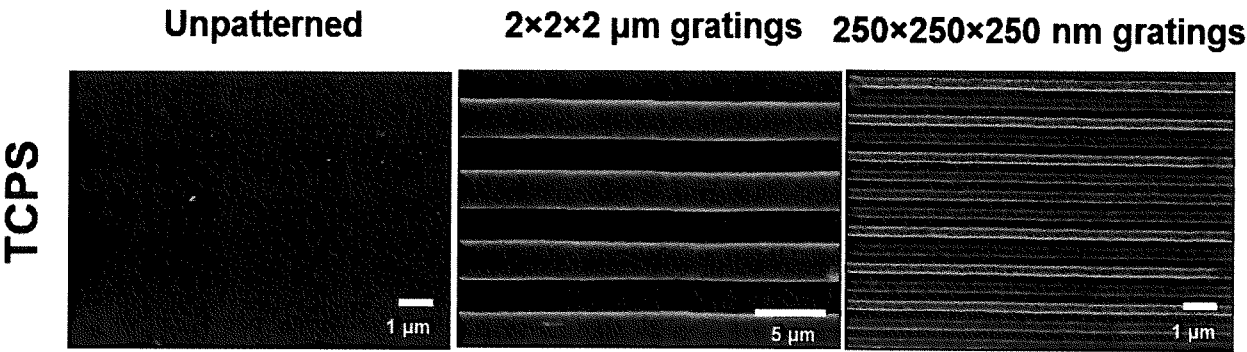


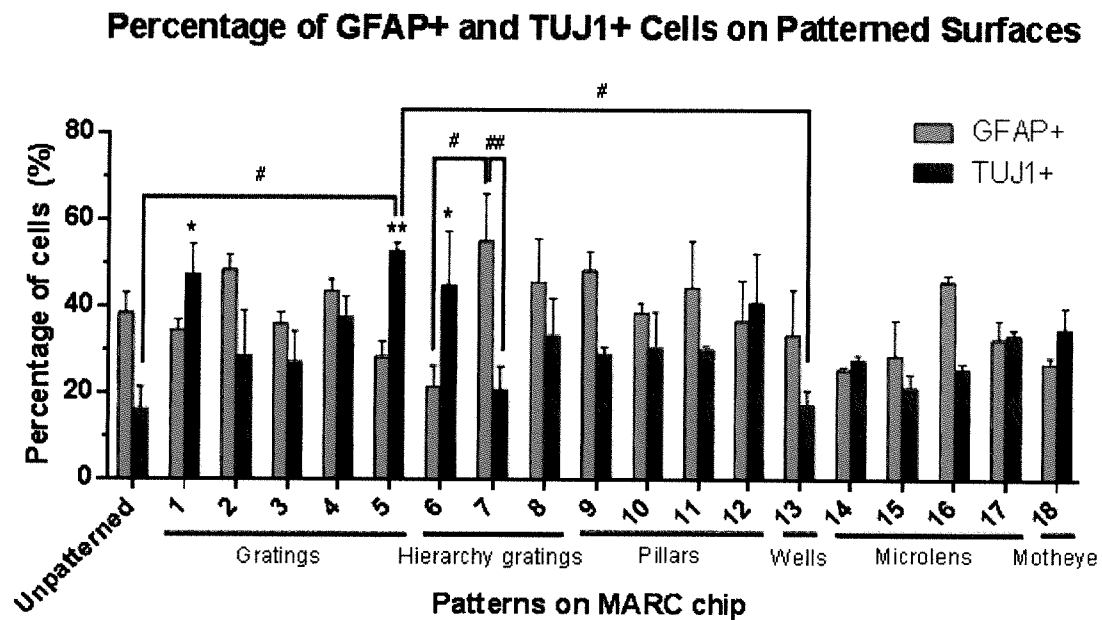
FIGURE 6



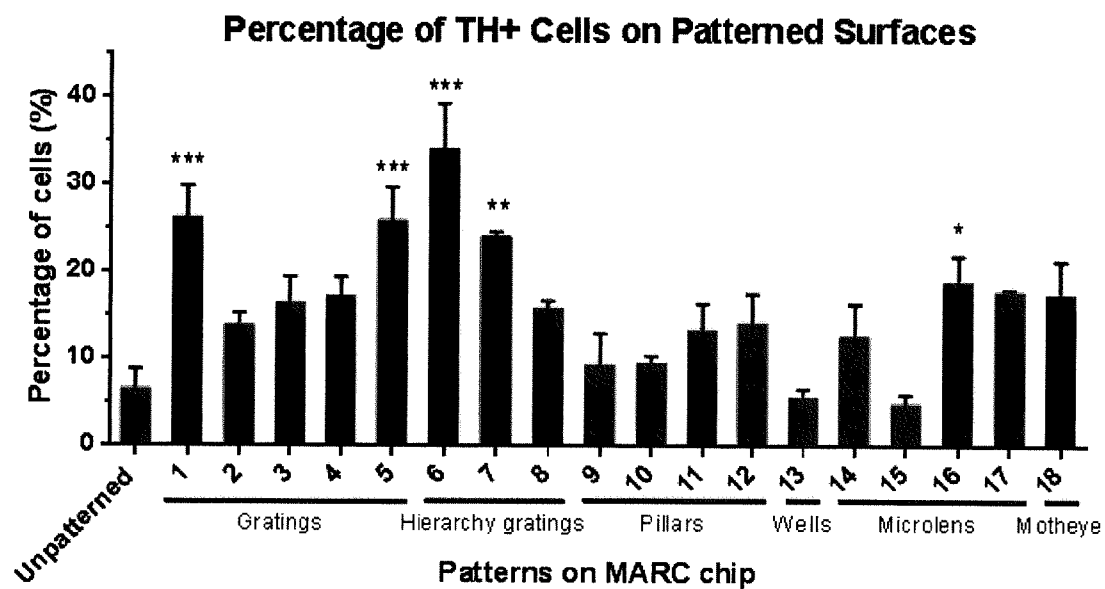
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FIGURE 7

A



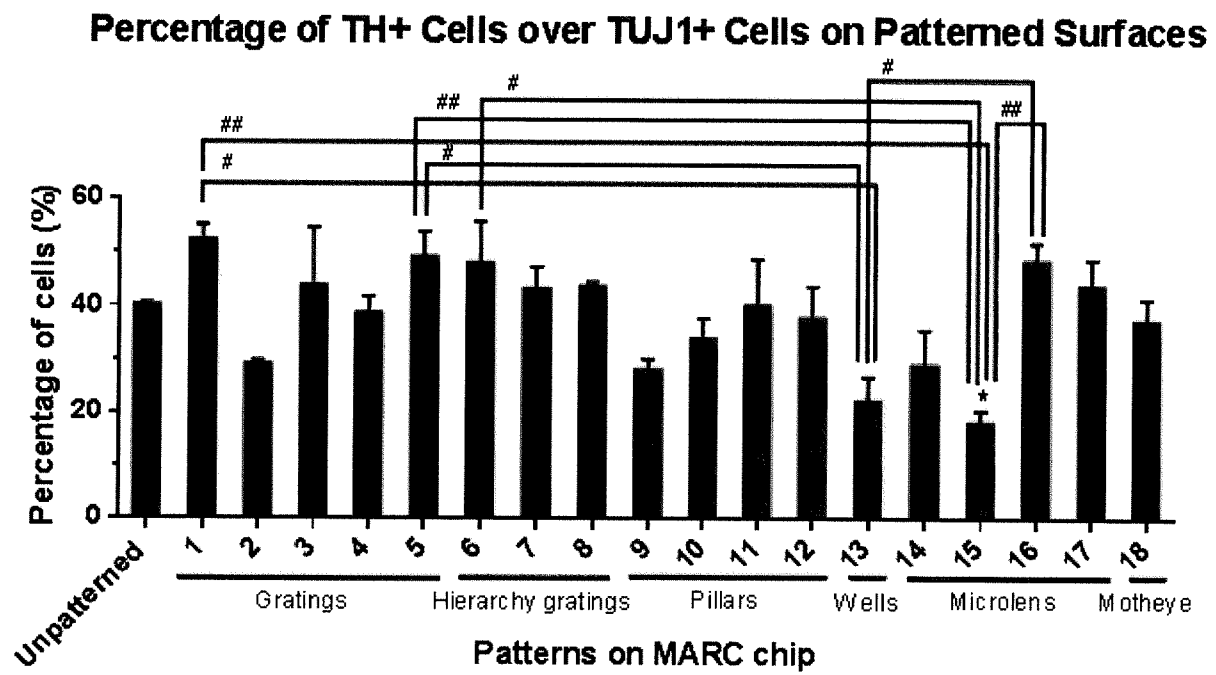
B



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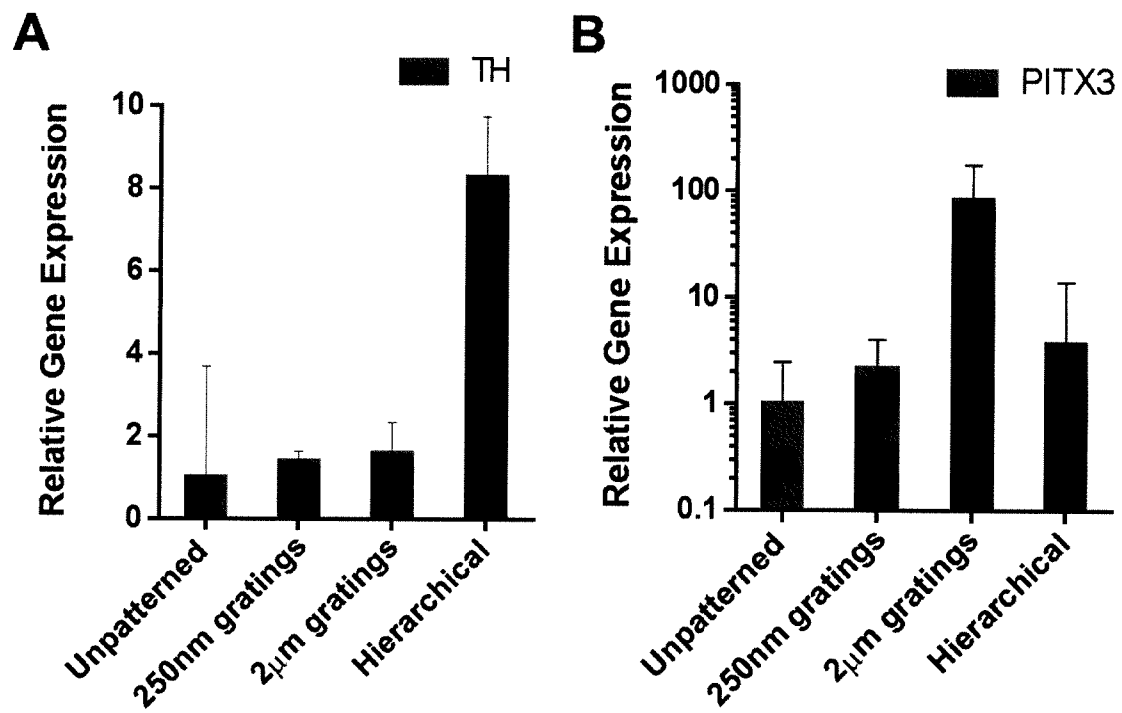
FIGURE 7 cont.

C



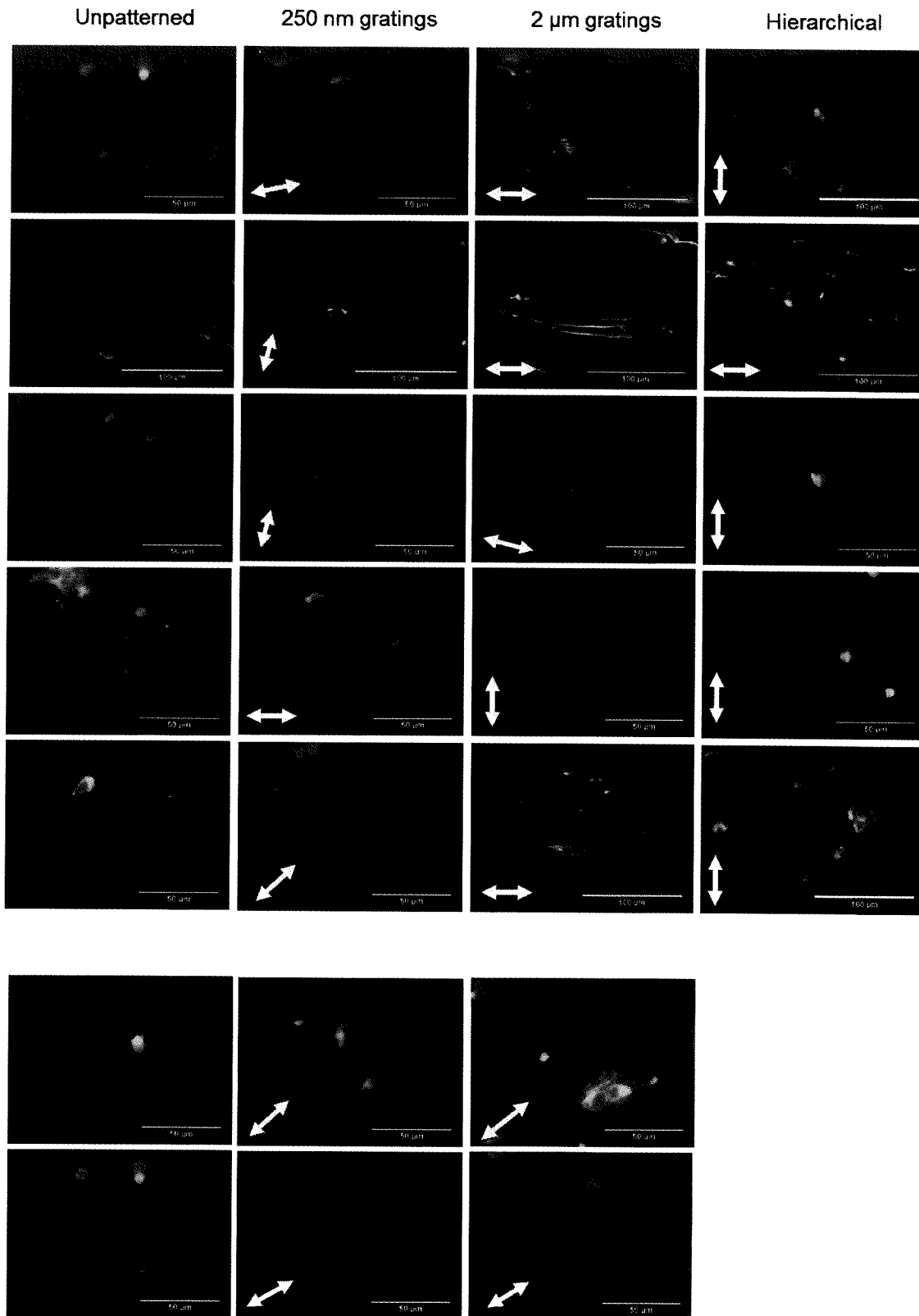
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FIGURE 8



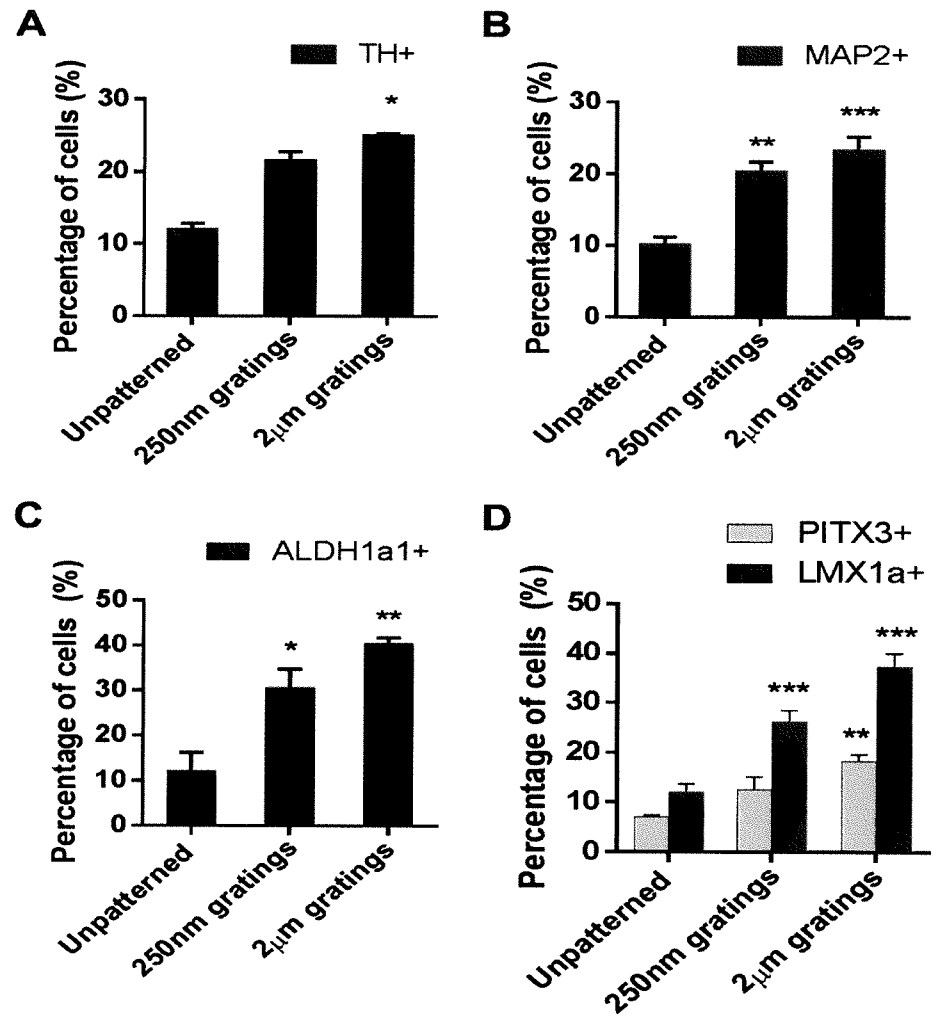
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FIGURE 9



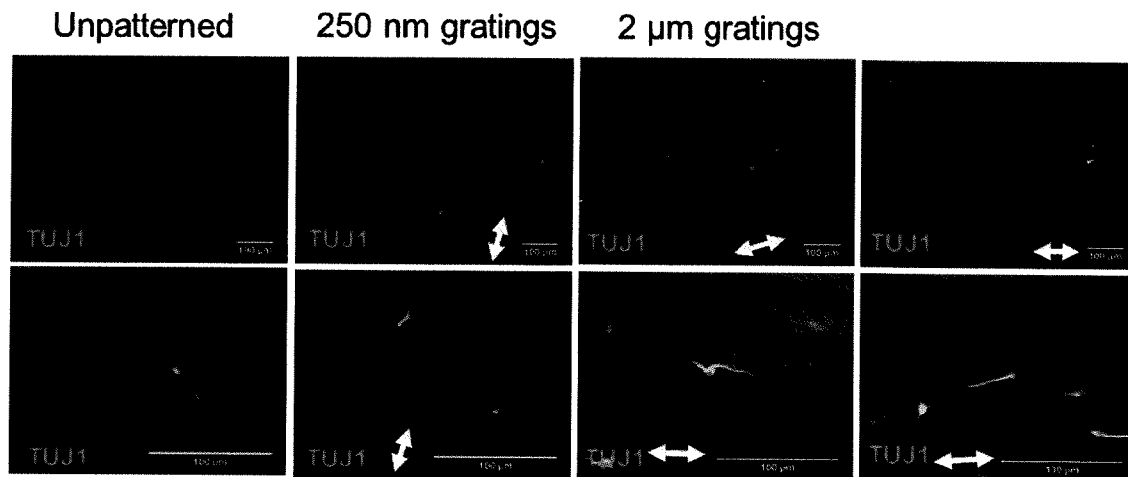
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FIGURE 10

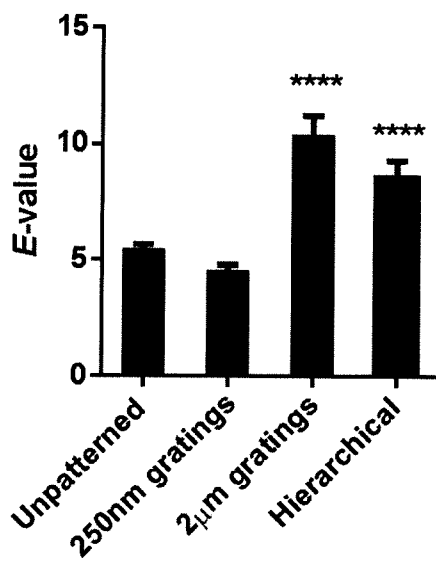


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FIGURE 11

A**B**

Elongation of Neurons

**C**

Alignment of Neurons

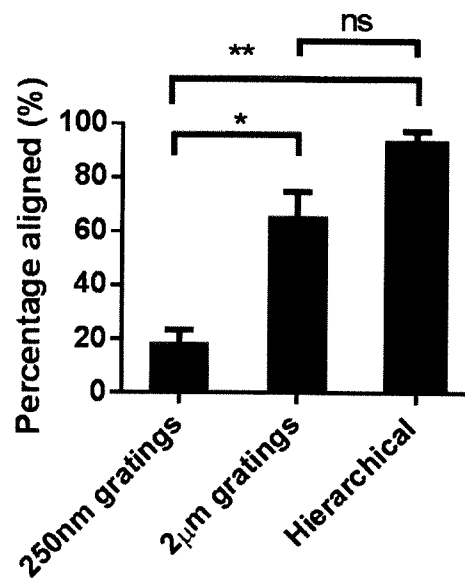
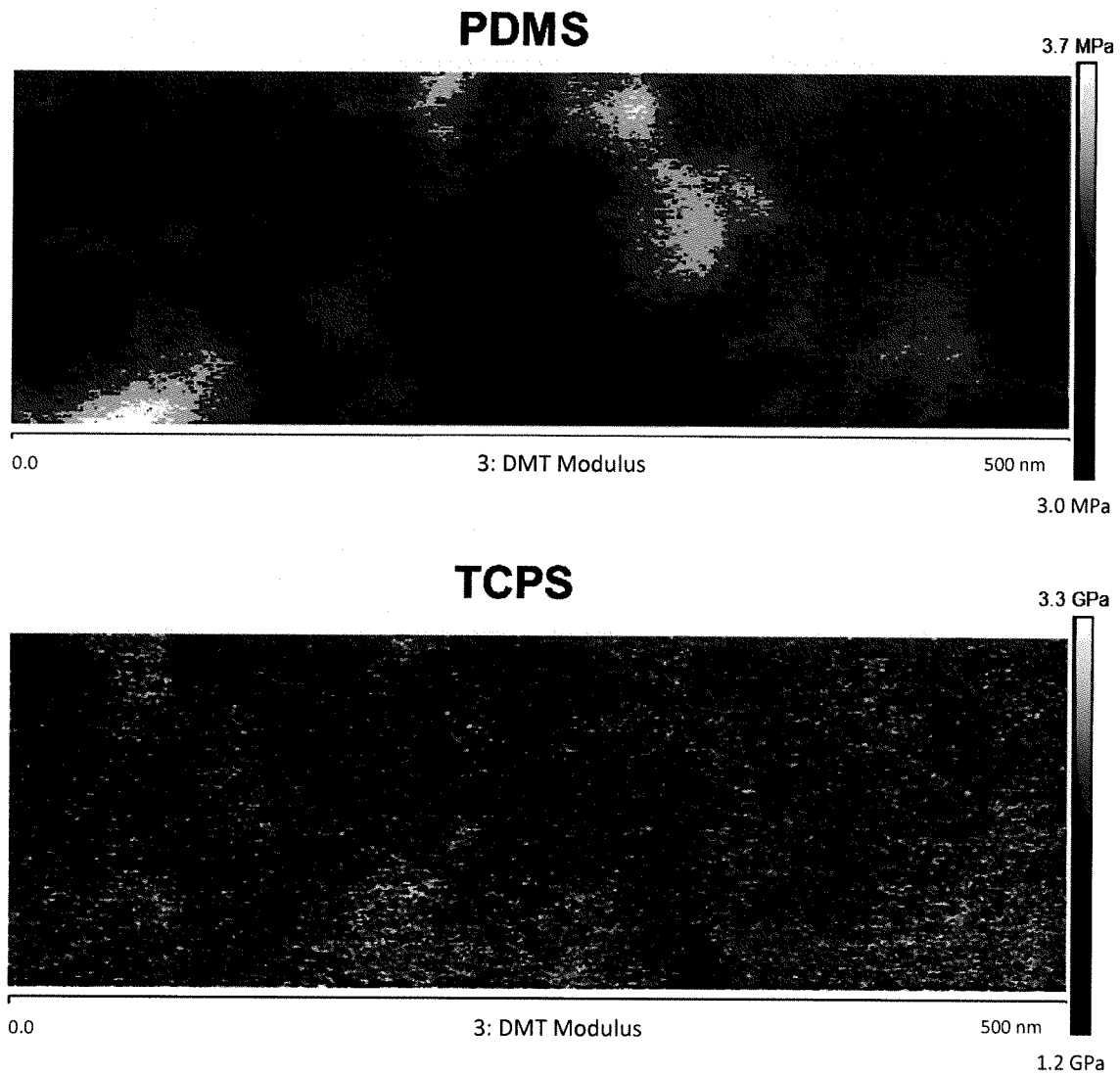


FIGURE 12



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FIGURE 13

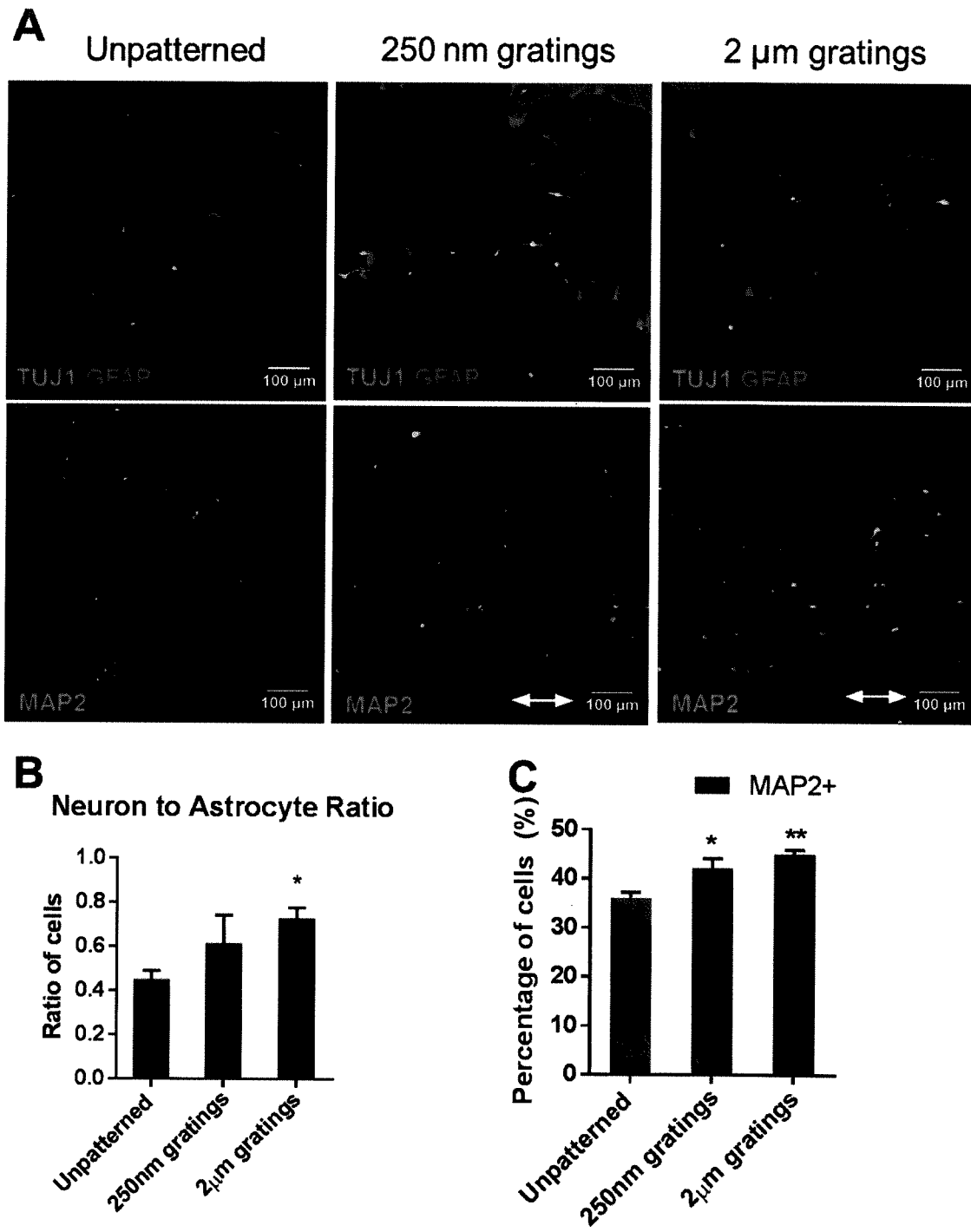
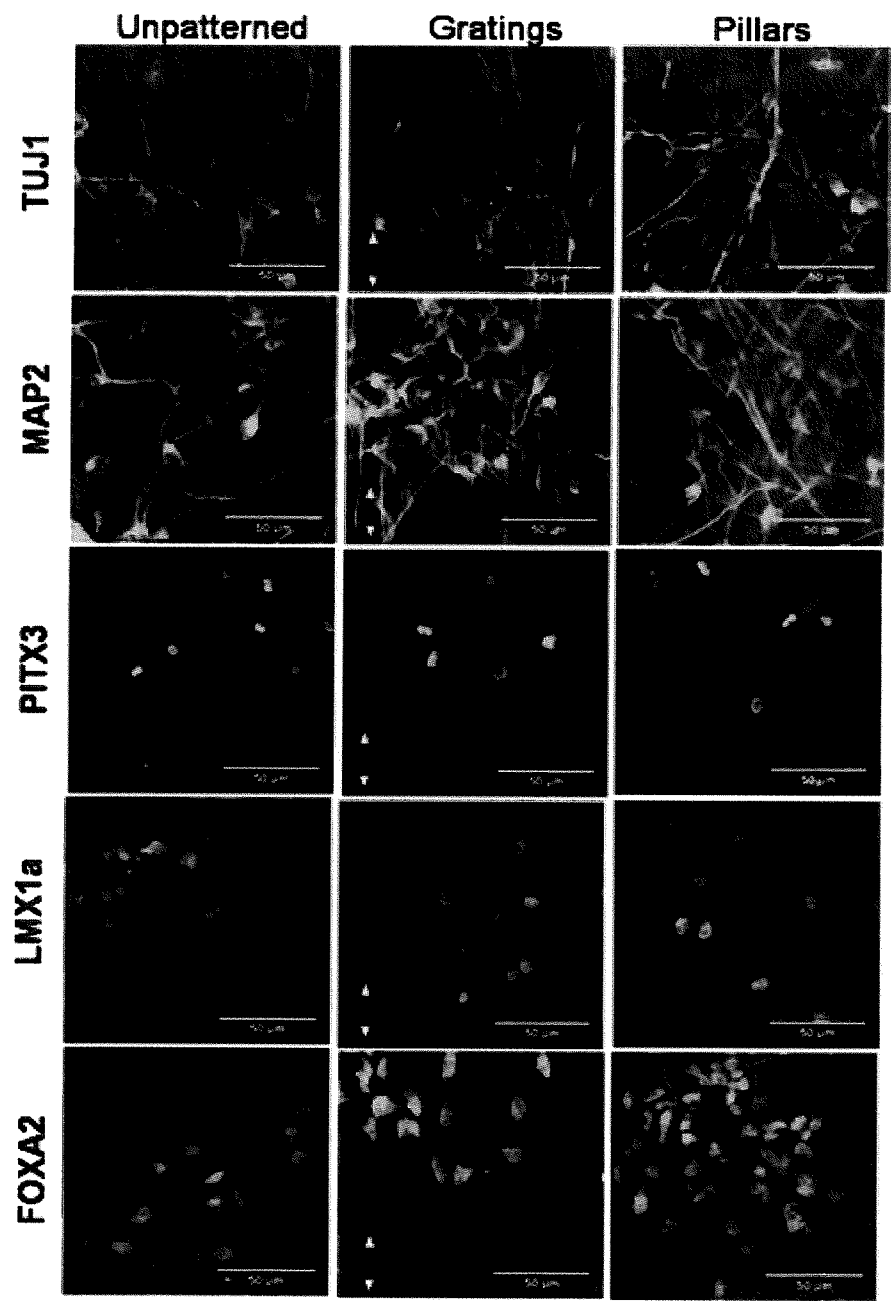


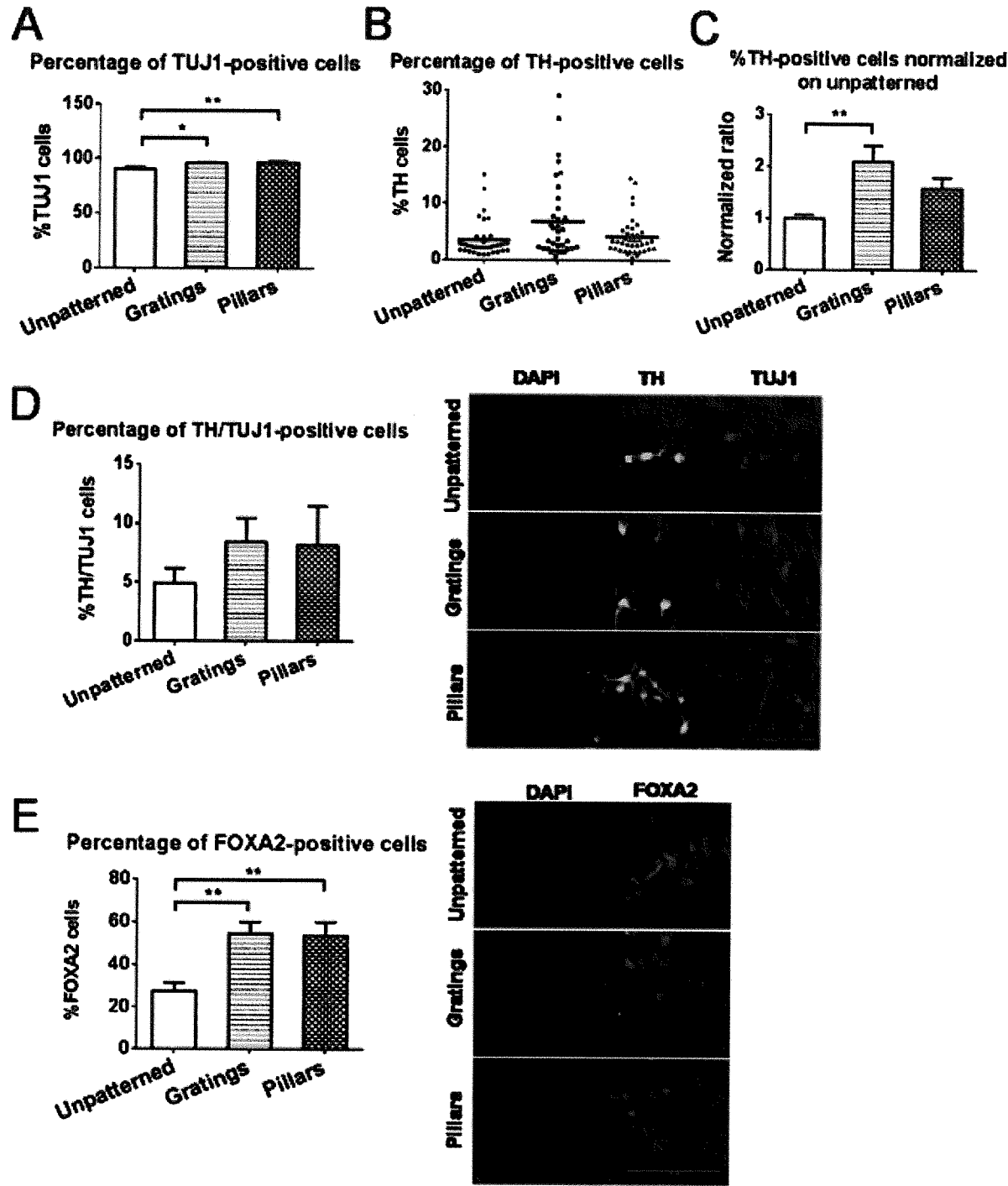
FIGURE 14



Scale bar = 50 μ m

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FIGURE 15



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FIGURE 16

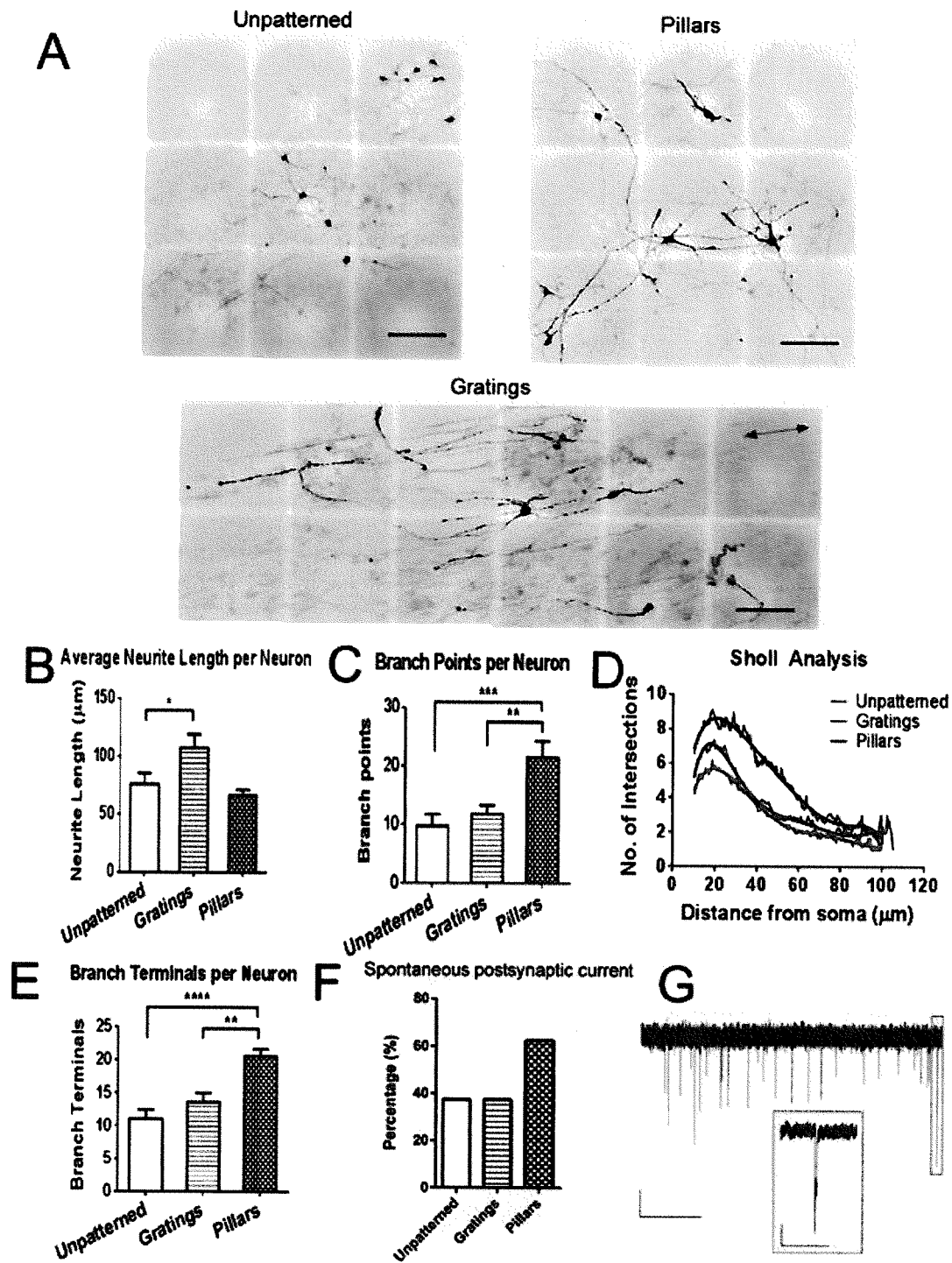


FIGURE 17

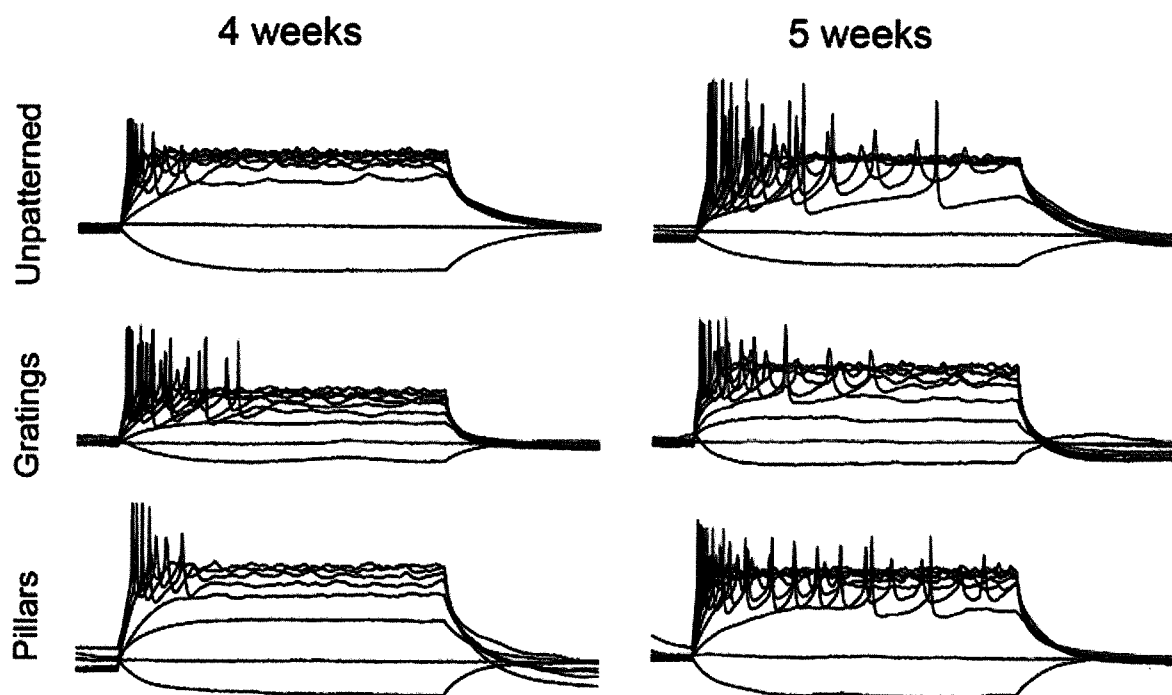
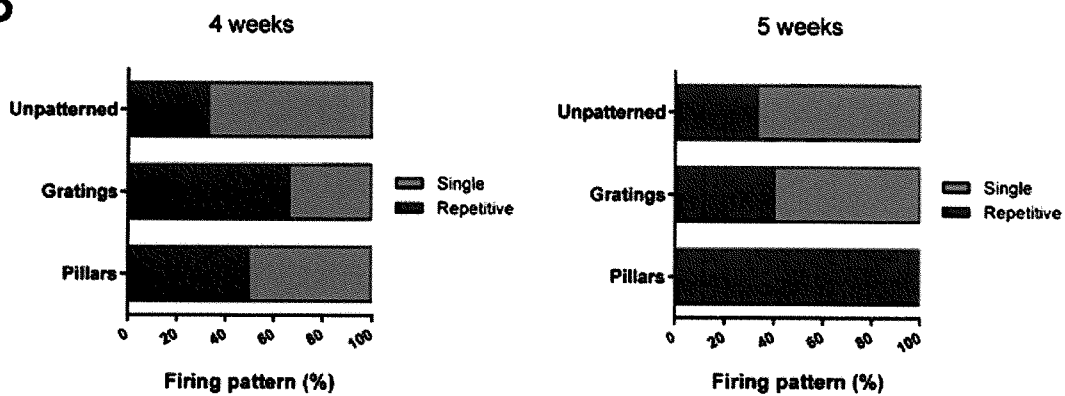
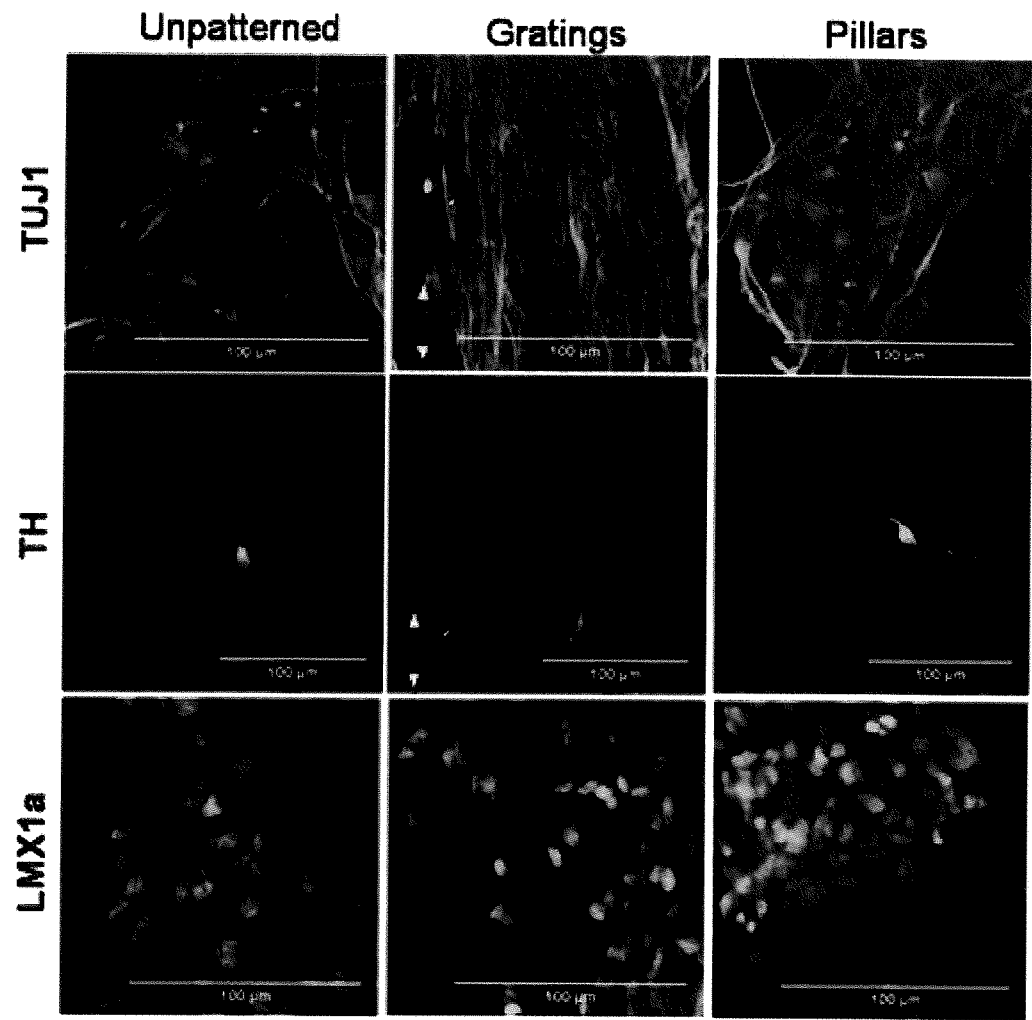
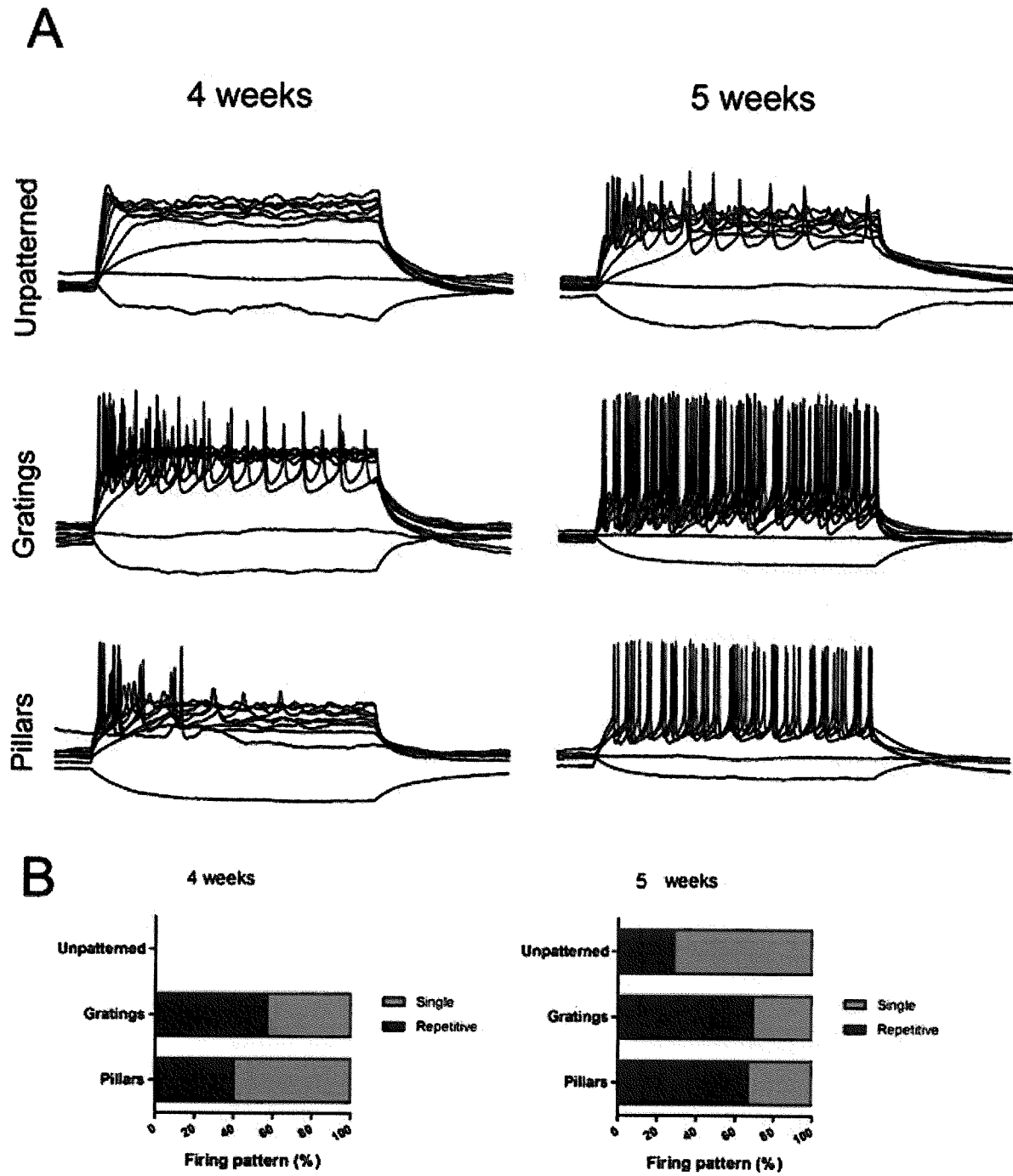
A**B**

FIGURE 18



Scale bar = 100μm

FIGURE 19



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2016/050128

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0793 (2010.01) B82Y 40/00 (2011.01) B01L 3/00 (2006.01) B81B 7/04 (2006.01) A61K 35/30 (2015.01)
C12Q 1/02 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPIAP, TXPEA, TXPEB, TXPEC, TXPEE, TXPEF, TXPEH, TXPEI, TXPEP, TXPES, TXPEPEA, TXPUSE0A, TXPUSE1A, TXPUSEA, TXPUSEB, TXPWOEA, MEDLINE, CAPLUS, BIOSIS, EMBASE, INSPEC, GOOGLE SCHOLAR & Keywords: neurogenesis, dopaminergic, neuronal cell, differentiation, enhance, substrate, PDMS, TCPS, grating, topography, hierarchical structure, nano-groove, micro-groove, pillar, post, extracellular matrix protein, Parkinson's Disease, treatment, drug screening, somatic cell, stem cell, progenitor cell, DMEM/F12, B27, N2, FGF-8, SHH, BDNF, GDNF, ascorbic acid, DAPT, laminin, fibronectin, poly-L-ornithine, SMAD, Noggin, CT9902, C12N 2533/30, C12N 2535, C12N 5/0068, C12N 2506/03, C12N 2506/45, C12N 5/0606, C12N 5/0607, C12N 5/0647, C12N 5/0696, and like terms, as well as Applicant & Inventor names.

INTESS, AusPat, PAMS & PUBMED (<http://www.ncbi.nlm.nih.gov/pubmed>): Applicant & Inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 June 2016	Date of mailing of the international search report 21 June 2016
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Alexander Theodossis AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 62832438

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2016/050128
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAN, K.K.B. <i>et al.</i> , 'Enhanced differentiation of neural progenitor cells into neurons of the mesencephalic dopaminergic subtype on topographical patterns', Biomaterials, 2015, Vol. 43, pages 32-43 (Epub 23 December 2014) Abstract; Sections 2.2, 2.3; Table 1; Figure 1; Sections 2.4; 2.5; para.3 of col.1 on p.36; Section 3.3; Figures 2-5; para.1-3 on p.32; Section 5	1-31
X	YOO, J. <i>et al.</i> , 'Nanogrooved substrate promotes direct lineage reprogramming of fibroblasts to functional induced dopaminergic neurons', Biomaterials, 2015, Vol. 45, pages 36-45 (Epub 13 January 2015) Abstract; Figures 1, 3, 6; para.1, 2 of col.1 on p.39; para.1 of col.1 on p.44; Figure 2A; Figure 1B; para.2, 3 of col.1 on p.44; Section 4	1-31
X	MOE, A.A.K. <i>et al.</i> , 'Microarray with micro- and nano-topographies enables identification of the optimal topography for directing the differentiation of primary murine neural progenitor cells', Small, 2012, Vol. 8, pages 3050-3061 (pages 1-12 in document retrieved online) & Supporting Information (available online) [retrieved from internet 3 June 2016] <URL: http://onlinelibrary.wiley.com/store/10.1002/sml.201200490/asset/supinfo/sml_201200490_sm_suppl.pdf?v=1&s=2264a60fd4ce5c2e94f89901d77412f3276d5a2b > Abstract; Figure 4; Supplementary Figure 4; Figure 2B; Table 1; para.3, 4 of col.1 on p.11; Figure 3B; para.8 of col.1 & para.1 of col.2 on p.11; para.1 of col.2 on p.3	1-31
X	KULANGARA, K. <i>et al.</i> , 'The effect of substrate topography on direct reprogramming of fibroblasts to induced neurons', Biomaterials, 2014, Vol. 35, pages 5327-5336 (pages 1-18 in document retrieved online) Abstract; Figure 2; para.3 on p.9; Section 2.1; Figure 1B-D; para.3 on p.6; Section 2.2	1-31
X	CHAN, L.Y. <i>et al.</i> , 'Temporal application of topography to increase the rate of neural differentiation from human pluripotent stem cells', Biomaterials, 2013, Vol. 34, pages 382-392 Abstract; Figures 1-7; Sections 2.1, 2.4, 2.5	1-31
X	YANG, K. <i>et al.</i> , 'Multiscale, hierarchically patterned topography for directing human neural stem cells into functional neurons', ACS Nano, 2014, Vol. 8, pages 7809-7822 Abstract; Figures 1, 2, 4, 7; para.5 of col.2 on p.7819	1-31
X	KIM, J.H. <i>et al.</i> , 'Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease', Nature, 2002, Vol. 418, pages 50-56 Abstract; para.2 of col.1 on p.55; Figures 1-5	1-31
X	PENG, J. <i>et al.</i> , 'Using human pluripotent stem cell-derived dopaminergic neurons to evaluate candidate Parkinson's disease therapeutic agents in MPP+ and rotenone models', Journal of Biomolecular Screening, 2013, Vol. 18, pages 522-533 Abstract; Figures 1, 3-5; Table 1	1-31
X	YANG, K. <i>et al.</i> , 'Nanotopographical manipulation of focal adhesion formation for enhanced differentiation of human neural stem cells', ACS Applied Materials & Interfaces, 2013, Vol. 5, pages 10529-10540 Abstract; Figures 1, 2, 4-6; Sections 2.1, 2.3	1-31
T	NGUYEN, A.T. <i>et al.</i> , 'From nano to micro: topographical scale and its impact on cell adhesion, morphology and contact guidance', Journal of Physics: Condensed Matter, 2016, Vol. 28: 183001 (Epub 12 April 2106) Whole document	1-31
Form PCT/ISA/210 (fifth sheet) (July 2009)		

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2016/050128
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Form PCT/ISA/210 (fifth sheet) (July 2009)		

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are 2 different inventions based on the following features that separate the claims into distinct groups:

Invention 1: Claims 1-17, 27-30 (in full), Claim 31 (in part). The feature of differentiating cells into neurons is specific to this group of claims.

Invention 2: Claims 18-26 (in full), Claim 31 (in part). The feature of gratings having width, spacing and height dimensions of 250nm to 2um is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a substrate patterned with nano- and/or micro-grating structures. However this feature does not make a contribution over the prior art because it is disclosed in:

D1: TAN, K.K.B. *et al.*, 'Enhanced differentiation of neural progenitor cells into neurons of the mesencephalic dopaminergic subtype on topographical patterns', Biomaterials, 2015, Vol. 43, pages 32-43 (Epub 23 December 2014)

D2: YOO, J. *et al.*, 'Nanogrooved substrate promotes direct lineage reprogramming of fibroblasts to functional induced dopaminergic neurons', Biomaterials, 2015, Vol. 45, pages 36-45 (Epub 13 January 2015)

D3: MOE, A.A.K. *et al.*, 'Microarray with micro- and nano-topographies enables identification of the optimal topography for directing the differentiation of primary murine neural progenitor cells', Small, 2012, Vol. 8 pages, 3050-3061 (pages 1-12 in document retrieved online)

D1 discloses substrates patterned with micro- to nano-scale gratings and hierarchical gratings (see Figure 1; Table 1).

D2 discloses substrates patterned with micro-scale and nano-scale grooved patterns, such as parallel gratings (see Figure 2A).

D3 discloses substrates characterised by a number of topographies, including: parallel gratings of 250nm width, 250nm spacing, 250nm height; parallel gratings of 2um width, 2um spacing, 2um height; and parallel 2um gratings with parallel or perpendicular 250nm hierarchical gratings within the base and grooves (see Figure 2B; Table 1).

Therefore in light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.