Macroporous heparin-based microcarriers allow long-term 3D culture and differentiation of neural precursor cells

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Abstract

Adult neurogenesis and the neurogenic niche in the dentate gyrus are subjects of much research interest. Enhancing our knowledge of this niche process and the role played by this unique microenvironment would further our understanding of plasticity and its relevance for cognition in health and disease. The complex three-dimensional (3D) nature of the niche microenvironment is poorly recapitulated in current cell culture experimental procedures. Neural precursor cells (NPCs) are cultured either on two-dimensional (2D) surfaces, where cells quickly reach confluency and passaging is required, or as 3D neurospheres, with the limitation of poor diffusion of nutrients and thus partial differentiation of cells over time. Herein, we culture NPCs on microscale scaffolds termed microcarriers, composed of poly(ethylene glycol) and heparin, designed to more closely represent the 3D environment of the neurogenic niche. The interconnected macroporous structure of the microcarriers allows NPCs to attach to their pore walls with subsequent continuous proliferation (analyzed up to 28 days) without formation of a necrotic core. Removal of basic fibroblast growth factor and epidermal growth factor from the culture medium results in differentiation of the NPCs. Unlike 2D culture, a high percentage of neurons was achieved on the microcarriers (22% MAP2 positive cells) indicating that these 3D microscale scaffolds give a more conducive environment for neuronal differentiation. Microcarrier culture of NPCs allows long-term cell expansion and better differentiation, which provides superior culture conditions for studying/modelling the neurogenic niche.

Introduction

Neural precursor cells (NPCs) are multipotent cells able to differentiate into cell types that belong to a neural lineage [1]. The population of NPCs that is located in the neurogenic niche of the adult hippocampal dentate gyrus possesses the potential of self-renewal and the ability to differentiate into mature neurons, astrocytes and oligodendrocytes [2]. The dentate gyrus acts as a critical regulatory gate in memory processing, thus plasticity in this region provides the requisite for network flexibility [3]. Researchers are trying to better understand the niche environment in order to explore the full capacity that NPCs hold for plasticity and potentially even endogenous repair [4, 5].

In order to expand, differentiate and study NPCs *in vitro*, one would ideally use a culture platform that mimics components of the niche microenvironment. Such a system would allow peptide mediated NPC attachment and a 3D space in which they can proliferate and form cell-to-cell contacts. In addition, spontaneous differentiation should be avoided, allowing the researchers to have temporal

control over such events. Currently, two culture methods are primarily in use, both of which fall short of the above-mentioned criteria. The first method is a 2D monolayer culture, on poly-D-lysine and laminin coated surfaces, and the second is the 3D culture of a dense sphere of aggregated cells, called neurosphere culture. 2D culture gives rise to a relatively pure/homogeneous population of cells, however, neuron yields are low and the lack of cell-to-cell contact in a 3-dimensional context poorly mimics the *in vivo* situation [6, 7]. In addition, due to the limited surface area for cell growth, the cells quickly reach confluency under proliferating conditions and have to be passaged, losing their existing microenvironment in the process. Neurosphere culture on the other hand, does not require regular passaging (though periodic dissociation is necessary) and allows 3D cell-to-cell contact. There are, however, intrinsic drawbacks of neurosphere culture such as heterogeneity of the cell population and spontaneous differentiation [8, 9]. This may be due to an uneven distribution of oxygen and other nutrients between the surface and the core of the neurosphere causing differential cell fates. For example, hypoxic areas have been imaged throughout neurospheres, in particular after longer culture periods and in the neurosphere core [10].

Biomaterial scaffolds and hydrogels could provide a more realistic environment for NPC culture, allowing more complex cell-matrix interactions [11-13]. Defined RGD-functionalized hydrogels have been synthesized from poly(ethylene glycol) and heparin, that allow NPCs to grow in a 3D matrix [7]. This hydrogel system gave a high degree of control over the matrix properties, whereby NPCs can be embedded in hydrogels of different stiffness, with the presence or absence of cleavable peptides that allow matrix remodeling. Such hydrogels represent a significant advancement of *in vivo* mimicry, however culture times over nine days could not be accomplished without heterogeneous nutrition supply occurring. Microcarriers or spherical beads have also been utilized as a means of culturing stem cells in bioreactors/spinner flasks [14-17]. However, such materials have a limited surface area for cell adhesion and do not really represent a 3D matrix environment. Despite these drawbacks, one group has used fibronectin and heparin functionalized chitosan microspheres as a surface for NPC growth and expansion, prior to transplantation into a model of traumatic brain injury [17]. Fibronectin was used to improve cell adhesion, since their data had shown it to be the best surface coating for allowing neurite outgrowth, and heparin was used for its ability to bind growth factors (in this case fibroblast growth factor (FGF-2)).

The aim of the present study was to investigate whether heparin-based microcarriers functionalized with a fibronectin-derived peptide sequence would support the adhesion and subsequent proliferation of NPCs. We have previously synthesized these microcarriers via cryogelation of a four-arm poly(ethylene glycol) (PEG) with heparin via carbodiimide chemistry [18]. The cryogelation process results in a macroporous structure [19], that is ideal for the culture of cells due to its high surface area to volume ratio. The presence of the highly sulfated glycosaminoglycan heparin in the structure also allows reversible attachment of heparin-binding growth factors. We thought that the combination of a matrix like structure that would bind and release mitogens from the medium, and a fibronectin-mimicking adhesion ligand (RGD peptide), could form a niche environment for proliferation of NPCs for extended periods of time (see Figure 1). Specifically we aimed to evaluate the possibility of culturing NPCs on the microcarriers for up to 28 days, assess the degree of spontaneous differentiation, and analyse the cell populations formed upon deliberate differentiation via growth factor withdrawal.

Materials and Methods

The materials and methods used for this study are detailed in the Supplementary Information. In brief, cryogel microcarriers were prepared in a manner similar to that reported previously [18] but functionalized with the RGD peptide motif. Hippocampal neural precursor cells (NPCs) extracted from

the dentate gyri of 6-8 week old C57BL/6 mice (as previously reported [20]) were cultivated either in 2D culture (40,000 cells seeded on laminin and poly-D-lysine coated 12 mm glass cover slips) or 3D culture. For 3D culture 500,000 NPCs were pipetted onto 1.5 mg of dry RGD-functionalized microcarriers in a centrifuge tube and topped up with 100 µL of NB + B27 medium. The cell loaded microcarriers were incubated for 1 h at 37 °C before being transferred to a 12-well plate for non-adherent culture (static culture) or a Sigmacote (Sigma-Aldrich) treated spinner flask (dynamic culture). Wells contained 1 mL of medium or spinner flasks (GPE Scientific Ltd – standard 25 mL flat bottom flask) contained 25 mL of medium (medium = NB + B27 media with growth factors EGF and bFGF as described above). A spin speed of 80 rpm was used, controlled by a Cimarec i Poly 15 stir plate (ThermoFisher). For proliferation conditions, half of the medium was changed every other day. Analysis was performed after 3, 7, 14 and 28 days. Differentiation of the 2D and 3D culture was induced by withdrawal of growth factors and samples were analyzed after a further 1, 3 , 6 and 14 days. For 3D culture experiments on Cultispher S (Sigma-Aldrich), the Cultisphers were first sterilized and dried according to the manufacturer's protocol, and used in an identical manner to the cryogel microcarriers.

Results

Microcarriers have a macroporous structure and are modified with RGD for cell adhesion

In order to provide a 3-dimentional substrate material for NPC attachment and growth, macroporous microcarriers were produced via a modification of a previously reported protocol [18]. This yielded microcarriers with a diameter distribution ranging from 110 µm to 550 µm (average diameter 325 µm, see Supplementary Figure S1a). The cryogelation process creates a macroporous structure, which was post functionalized with a fibronectin-derived RGD peptide sequence. The microcarriers therefore possess a large surface area for cell adhesion and growth. The porous structure can be visualized by scanning electron microscopy (dry microcarrier) and confocal fluorescence microscopy (hydrated microcarrier) as shown in Figure 1. The microcarriers used in these studies have an average pore size of 37 µm (standard deviation = 10 µm, see Supplementary Figure S1b). The addition of RGD motifs to biomaterials has been well documented to improve cell adhesion [21] and so was added to these microcarriers. Qualitative assessment of NPC adhesion after three days of culture (Supplementary Figure S2) showed that RGD was necessary for good cell attachment and subsequent growth.

Neural precursor cells can be cultured on microcarriers and maintain viability for 4 weeks

Mouse dentate gyrus NPCs were successfully cultured on the cryogel microcarriers. Unlike 2D culture, where NPCs cannot be cultured beyond confluency without being passaged, microcarrier culture of NPCs could be carried out for at least 28 days (longest time analyzed). The live/dead cell assay was used to analyze the viability of the NPCs cultured on the microcarriers at four time points. Figure 2 shows that for all time points an even spread of calcein stained live cells can be seen throughout a 40 µm z-stack through the microcarriers. Quantification of the number of nuclei (counterstained with Hoechst 33342) shows a drop in cell number at day 7, but a revival and growth until day 28 (fold increase in nuclei between day 3 and day 28: 2.9) with little change in the percentage of dead cells (Figure 2a). Thus, in contrast to conventional 2D culture the cells do not have to be passaged during this extended culture period.

In order to provide a comparison of the microcarriers with a commercially available cell carrier system, a separate study was performed whereby NPCs were either loaded to the microcarriers or to the porous gelatin-based Cultispher S system. Supplementary Figure S3 shows a tendency for a greater number of NPCs to be obtained on microcarriers compared to Cultisphers though this did not reach a

statistically significant difference after either 3 or 7 days in culture. Interestingly, however, a slightly reduced percentage of dead cells was observed when NPCs were cultured on microcarriers rather than Cultisphers ($P<0.0001$ for day 3 and $P<0.5$ for day 7).

We noticed that after 28 days in culture, some of the NPC-loaded microcarriers formed clusters (as shown in Supplementary Figure S4). This effect was not observed in the shorter culture periods, indicating that this is not a result of how the cells are seeded on the scaffold structure, but rather occurs over time. A group of dead cells could be seen in the center of this cluster, which led us to investigate: a) whether dynamic spinner flask culture would provide better culture conditions (other studies have adopted spinner flask cultures to keep the microcarriers in motion [18]), and b) whether the size of the microcarriers used influenced the number of nuclei or the number of dead cells on the microcarriers. In this study we found no additional benefit of using dynamic culture, but rather a reduced cell number on both microcarriers and CultiSphers and a greater percentage of dead cells (Supplementary Figure S5), though no further optimization of dynamic conditions was performed. Instead, static culture was then used to quantify of the number of nuclei and the number of dead cells with respect to the diameter of the corresponding microcarrier (Supplementary Figure S6). At day 3 and day 28 there was a slight tendency for larger microcarriers to contain more cells, however, there was no pronounced overall trend. The percentage of dead cells did not increase with increasing microcarrier diameter or time of culture (Supplementary Figure S7) indicating that the core is not becoming devoid of nutrients. The open macroporous microcarrier structure allows cell growth on the surface of the struts (pore walls) and thus not to restrict oxygen flow to the core (unlike the tightly packed mass of cells in neurosphere culture [10]). Once NPCs cluster and become so dense that the microcarriers connect, this obviously does not hold true any longer and a necrotic core forms as shown in Supplementary Figure S4. However, an insufficient number of connected clusters was found for quantitative analysis.

Self-renewal and multipotency during extended culture on the microcarriers

Qualitative analysis of nestin expression was performed (Figure 3a), together with quantification of the number of cells expressing Sox2 and PCNA (proliferative precursors) which was compared to quantification of differentiated cells (which express MAP2 and GFAP - early neuronal and astrocytic markers respectively) (Figure 3b). The glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in glia cells, typically astrocytes, in the CNS [22]. Nevertheless, it has to be considered that also neural precursors express GFAP [23], which can be observed by a faint signal in Figure 3. Under these culture conditions, in the presence of the growth factors EGF and bFGF, very little spontaneous differentiation is taking place, allowing the expansion of NPCs on the microcarriers for a month. Figure 3c shows microcarriers that have clustered together after 28 days in culture, exhibiting a dense and even spread of both nestin positive and Sox2 positive cells, indicating their capacity for self-renewal. In addition to the long cell culture experiments we analyzed whether NPCs could be removed from the microcarriers. Two cell dissociation solutions, accutase® and accumax™, successfully removed the NPCs from the microcarriers leaving them largely devoid of cells (Supplementary Figure S8). Cell viability analysis (both live/dead analysis and trypan blue exclusion) revealed over 95% of the cells were viable which could be seeded for further use (Supplementary Figure S8d).

NPC differentiation potential in microcarrier culture

Current NPC differentiation protocols typically give a low neuron yield. We wanted to see whether greater numbers of neurons could be obtained after differentiation of NPCs in the 3D environment provided by the microcarriers. Removal of the growth factors from the culture media is a method

typically employed to initiate differentiation of NPCs *in vitro*. Based on previous reports, NPC culture for six days in growth factor free media drive cells into the expression of the first differentiation markers [24]. This protocol was used and again the live/dead assay was carried out before and after six days of differentiation for qualitative analysis of cell survival under these conditions (Figure 4). Many live cells can be observed which spread over the microcarriers at both 7 and 28 days, and also after another 6 days of differentiation. Although the differentiation procedure resulted in a loss of nuclei and an increase in dead cell fraction (Figure 4c), overall the 3D microcarrier culture of NPCs for 28 days prior to differentiation gave rise to a higher number of nuclei and lower percentage of dead cells than the standard 2D culture with subsequent differentiation. Again, the markers MAP2 and GFAP were used in comparison to nestin, Sox2 and PCNA for analysis of differentiation (Figure 5).The number of cells expressing these markers was quantified for a range of culture times and differentiation times as shown in Figure 5b. Culturing the NPCs on the microcarriers under proliferation conditions for 3, 7 and 28 days resulted in over 80% of cells expressing Sox2 and over 60% of the cells expressing PCNA. Growth factor removal led to the differentiation of NPC culture, which resulted in a reduced expression of proliferation and precursor state makers and an increased expression of the differentiation markers for neurons (MAP2) and astrocytes (GFAP). Thus, 3 days of proliferation and 3 days in differentiation conditions resulted in a reduction in the percentage of PCNA positive cells to 50% ±16%. Further, the percentage of MAP2 positive neurons increased significantly to 13% ±7%, with a corresponding rise in astrocytes to 12% ±6%. Seven days of culture followed by differentiation only gave rise to increased astrocyte populations, however the largest change was observed when differentiating NPCs for 6 days after 28 days of microcarrier culture. Under these conditions the percentage of MAP2 positive neurons rose to 22% ±2%, and the percentage of astrocytes increased from to 17% ±3%). These values were higher than those achieved in our control group of NPCs under 2D culture conditions with 6 days of differentiation (neurons: 8% ± 1%, and astrocytes: 11% ± 4%).

Discussion

In general, differentiation of NPCs by removal of FGF-2 and EGF from the medium typically results in astrocytes and a low neuron yield. For example Guo et al., stated that differentiation of NPC neurospheres derived from the dentate gyrus typically yields 19% GFAP positive astrocytes and 12% Tuj-1 positive neurons [25]. Such low yields of neurons makes studies related to neurogenesis mechanisms difficult. Substantial effort has been made to improve the yield of neurons in both 2D and 3D neurosphere culture in particular through co-culturing NPCs with other cells from the neurogenic niche that may provide support during differentiation [6]. Differentiation of NPCs grown in 2D culture in the presence of (but not in direct contact with) either astrocytes, pericytes or endothelial cells generally resulted in an increased percentage of cells entering neural lineage. This beneficial effect of culture was not observed for 3D neurosphere culture where the maximum percentage of Mab2ab positive neurons across all culture conditions reached ~15%. These studies have shown that other cell types in the neurogenic niche may guide the differentiation of NPCs, though further support is perhaps required. This led us to the idea of using microcarriers to culture and differentiate NPCs on in order to try and mimic a three-dimensional *in vivo* microenvironment. Rather than culturing cells on the outside of spheres [17], we hypothesized that macroporous microcarriers would provide a large surface area on which to culture NPCs. In this way they would maintain cell-cell contact and could multiply to fill the pores (compare Figure 2) in a manner more similar to 3D neurosphere culture. A recent study has shown that NPCs can be cultured within a hydrogel matrix and that cell adhesion is highly dependent on the matrix properties [7]. Neuronal differentiation was not assessed in that study, but such biomaterials may also play a useful role in supporting NPCs through the differentiation process. A major drawback of that technique stems from the poor diffusion of antibodies through the

matrix, making subsequent cell characterization difficult. In contrast, microcarrier based culture allowed good visualization of the cells in culture both via live/dead (calcein/propidium iodide) staining and immunocytochemistry.

2D culture of mouse NPCs sourced from the dentate gyrus typically yields a very low number of neurons (approximately 1 - 2%) when no additional niche components are supplemented [6]. This study confirmed low neuron yield in 2D culture but also highlighted the large cell death post differentiation (61% dead cells). However, the use of microcarriers for 28 day NPC culture and subsequent differentiation can give a much higher yield of neurons (22% MAP2 neurons), and, although still sub-optimal, lower rates of cell death (46%). Microcarrier-based culture techniques therefore outperform current methods of NPC culture. This rise in neuronal differentiation is particularly useful for functional analysis of the differentiated cells as well as for further co-culture investigations to gain a better understanding of the niche and cell-to-cell communication/dependencies.

Limitations of this study and future directions

One limitation of this study is the lack of control over the sizes of individual microcarriers. Our data do not indicate that the microcarrier diameter plays an important role in cell death, however, having microcarriers of the same diameter would reduce the variables in the study (especially if culture times longer than 28 days are envisaged) and could lead to greater consistency in results. Thus, ongoing work deals with producing such monodisperse microcarriers by means of droplet microfluidics.

The use of microcarriers allows sustained long-term proliferation of NPCs. Unlike current NPC 2D culture protocols, that require passaging of the proliferating cells every 2-3 days, the microcarrier-based cultures can be kept for at least 28 days without passaging. In addition, since the microcarrier scaffolds have a macroporous structure, it is unlikely that necrotic pockets will form giving rise to uneven cell populations (as can be observed in neurosphere culture[8]). It appears from qualitative analysis that the cells are well spread across the microcarriers in both a differentiated and undifferentiated state (Figures 2 and 4). By removing nutrient heterogeneity (a problem in neurosphere culture), microcarrier-based 3D culture could open up investigations into developing a 3D model of the dentate gyrus niche environment, which more closely represents the *in vivo* environment than current culture methods. For example, one could envisage the co-culture of multiple cell types as has previously been explored in our research group [6].

Conclusion

In summary we have investigated the use of heparin-based microscale spherical macroporous scaffolds obtained by cryogelation technology (herein termed microcarriers) for the long-term culture of neural precursor cells. We have shown that the cells can proliferate on the microcarriers and hold markers of stemness over a period of 28 days. Withdrawal of growth factors from the medium results in differentiation of the cells as indicated by the presence of a high number of neurons and astrocytes. This culture technique allows expansion of NPCs and controlled differentiation for further use in modelling/studying the neurogenic niche.

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Author Contributions:

BN and CW conceived the idea. FH, FE, HH, DE, and BN performed the experiments and analysis. PW, FE, GK, CW and BN critically analyzed the data and assisted in experimental design. All authors contributed to writing the manuscript.

Declaration of Interest:

The authors declare no conflicts of interest

Data Availability:

The raw/processed data required to reproduce these findings are available upon request.

Supplementary Information: Supplementary Figures S1-S8 are available online

References

[1] Gage FH, Kempermann G, Palmer TD, Peterson DA, Ray J. Multipotent progenitor cells in the adult dentate gyrus. Journal of Neurobiology. 1998;36:249-66.
[2] Kim YH, Chung JI, Woo HG, Jung YS, Lee SH, Moon CH, et al. Differential regulation of proliferation and differentiation in neural precursor cells by the Jak pathway. Stem Cells. 2010;28:1816-28.
[3] Kempermann G. Seven principles in the regulation of adult neurogenesis. European Journal of Neuroscience. 2011;33:1018-24.
[4] Bond Allison M, Ming G-l, Song H. Adult mammalian neural stem cells and neurogenesis: Five decades later. Cell Stem Cell. 2015;17:385-95.
[5] Lin R, Iacovitti L. Classic and novel stem cell niches in brain homeostasis and repair. Brain Research. 2015;1628:327-42.
[6] Ehret F, Vogler S, Kempermann G. A co-culture model of the hippocampal neurogenic niche reveals differential effects of astrocytes, endothelial cells and pericytes on proliferation and differentiation of adult murine precursor cells. Stem Cell Research. 2015;15:514-21.
[7] Vogler S, Prokoph S, Freudenberg U, Binner M, Tsurkan M, Werner C, et al. Defined geldrop cultures maintain neural precursor cells. Scientific Reports. 2018;8:8433.
[8] Jensen JB, Parmar M. Strengths and limitations of the neurosphere culture system. Molecular Neurobiology. 2006;34:153-61.
[9] Bez A, Corsini E, Curti D, Biggiogera M, Colombo A, Nicosia RF, et al. Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization. Brain Research. 2003;993:18-29.
[10] Dmitriev RI, Zhdanov AV, Nolan YM, Papkovsky DB. Imaging of neurosphere oxygenation with phosphorescent probes. Biomaterials. 2013;34:9307-17.
[11] Gelain F, Bottai D, Vescovi A, Zhang S. Designer self-assembling peptide nanofiber scaffolds for adult mouse neural stem cell 3-dimensional cultures. PLOS ONE. 2006;1:e119.
[12] Tsurkan MV, Wetzel R, Pérez-Hernández HR, Chwalek K, Kozlova A, Freudenberg U, et al. Photopatterning of multifunctional hydrogels to direct adult neural precursor cells. Advanced Healthcare Materials. 2015;4:516-21.

[13] Lee Y-B, Polio S, Lee W, Dai G, Menon L, Carroll RS, et al. Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture. Experimental Neurology. 2010;223:645-52.
[14] Abranches E, Bekman E, Henrique D, Cabral J. Expansion of mouse embryonic stem cells on microcarriers. Biotechnology and Bioengineering. 2007;96:1211-21.
[15] Fernandes A, Fernandes T, Diogo M, da Silva CL, Henrique D, Cabral J. Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system. Journal of Biotechnology. 2007;132:227-36.
[16] Qiu L, Lim YM, Chen AK, Reuveny S, Oh SK, Tan EK, et al. Microcarrier-expanded neural progenitor cells can survive, differentiate, and innervate host neurons better when transplanted as aggregates. Cell Transplantation. 2016;25:1343-57.
[17] Skop NB, Calderon F, Cho CH, Gandhi CD, Levison SW. Optimizing a multifunctional microsphere scaffold to improve neural precursor cell transplantation for traumatic brain injury repair. Journal of Tissue Engineering and Regenerative Medicine. 2016;10:E419-E32.
[18] Newland B, Welzel PB, Newland H, Renneberg C, Kolar P, Tsurkan M, et al. Tackling cell transplantation anoikis: An injectable, shape memory cryogel microcarrier platform material for stem cell and neuronal cell growth. Small. 2015;11:5047-53.
[19] Eigel D, Zoupi L, Sekizar S, Welzel PB, Werner C, Williams A, et al. Cryogel scaffolds for regionally constrained delivery of lysophosphatidylcholine to central nervous system slice cultures: A model of focal demyelination for multiple sclerosis research. Acta Biomaterialia. 2019.
[20] Walker TL, Kempermann G. One mouse, two cultures: Isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. JoVE. 2014:e51225.
[21] Huettner N, Dargaville TR, Forget A. Discovering cell-adhesion peptides in tissue engineering: Beyond RGD. Trends in Biotechnology. 2018;36:372-83.
[22] Lyck L, Dalmau I, Chemnitz J, Finsen B, Schrøder HD. Immunohistochemical markers for quantitative studies of neurons and glia in human neocortex. Journal of Histochemistry & Cytochemistry. 2008;56:201-21.
[23] Liu Y, Namba T, Liu J, Suzuki R, Shioda S, Seki T. Glial fibrillary acidic protein-expressing neural progenitors give rise to immature neurons via early intermediate progenitors expressing both glial fibrillary acidic protein and neuronal markers in the adult hippocampus. Neuroscience. 2010;166:241-51.
[24] Babu H, Claasen J-H, Kannan S, Rünker AE, Palmer T, Kempermann G. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Frontiers in Neuroscience. 2011;5:89.
[25] Guo W, Patzlaff NE, Jobe EM, Zhao X. Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. Nature Protocols. 2012;7:2005.

Figures

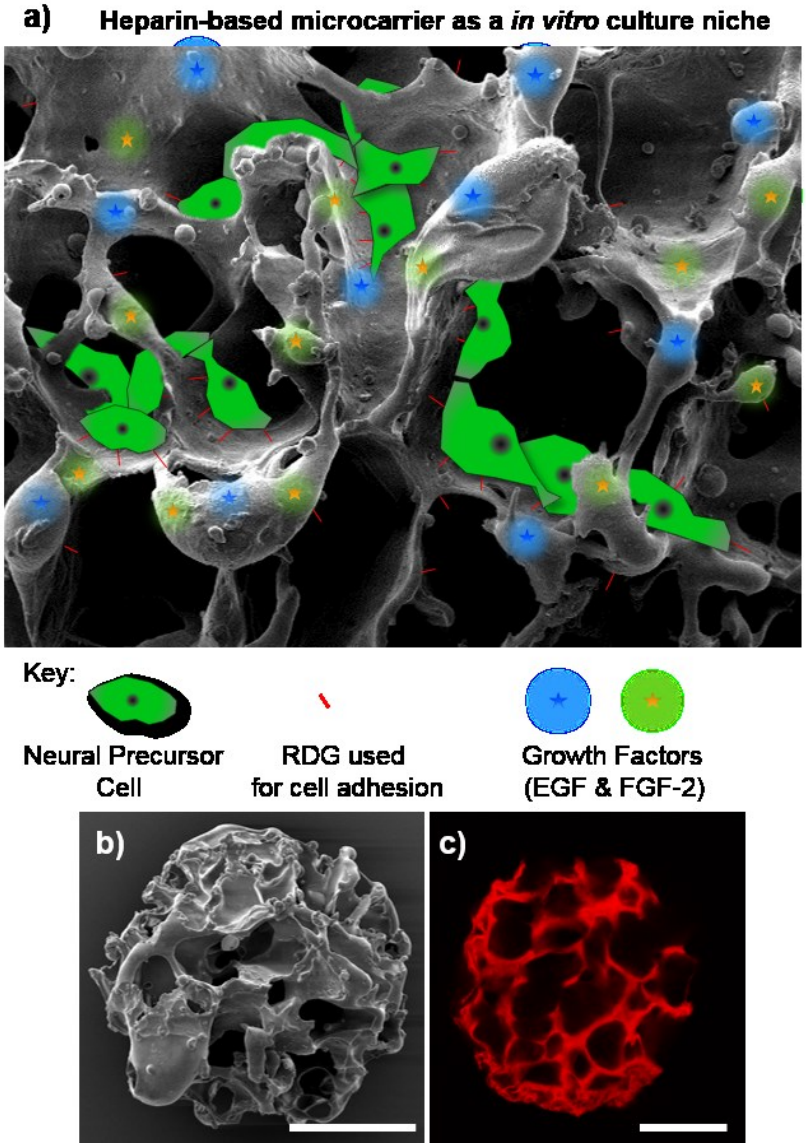

Figure 1. **The microcarriers are synthesized via a cryogelation process, which leaves them with a macroporous structure.** a) Schematic depiction of NPCs cultured on the microcarriers comprised of poly(ethylene glycol), heparin and the RGD peptide. b) Scanning electron microscope image of a microcarrier under vacuum. b) confocal laser scanning microscopy image of a hydrated microcarrier in cell culture medium (scale bars represent 50 µm).

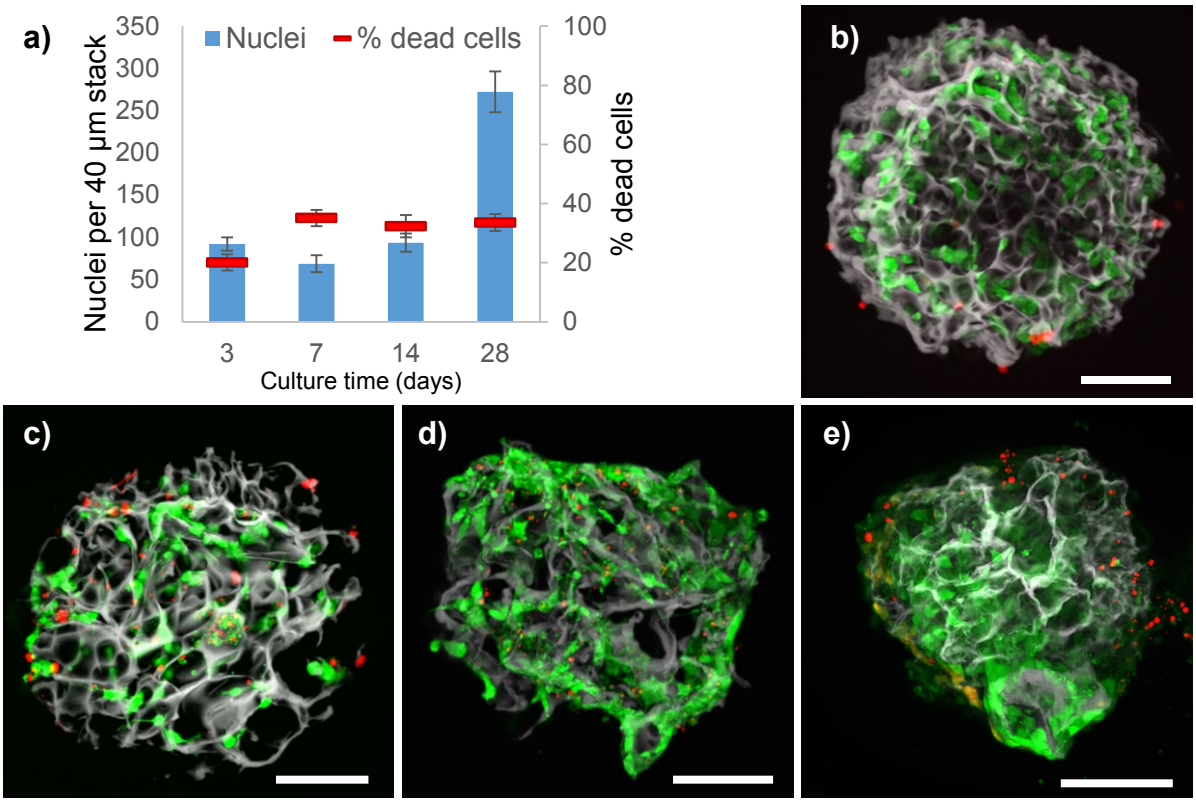

Figure 2: Live/Dead fluorescence staining of NPC culture on microcarriers (MC) in proliferative conditions for 3, 7, 14 and 28 days. a) Quantification of the number of nuclei in a 40 µm z plane of the microcarrier with corresponding percentage of dead cells (n=3, 30 microcarriers per time point, total number of cells analyzed = 15,340, error bars represent ± standard error of the mean). Representative images of calcein positive live cells (green) and propidium iodide positive dead cells (red) after b) 3 days, c) 7 days, d) 14 days and e) 28 days of culture on the microcarriers (white). Scale bars represent 100 µm.

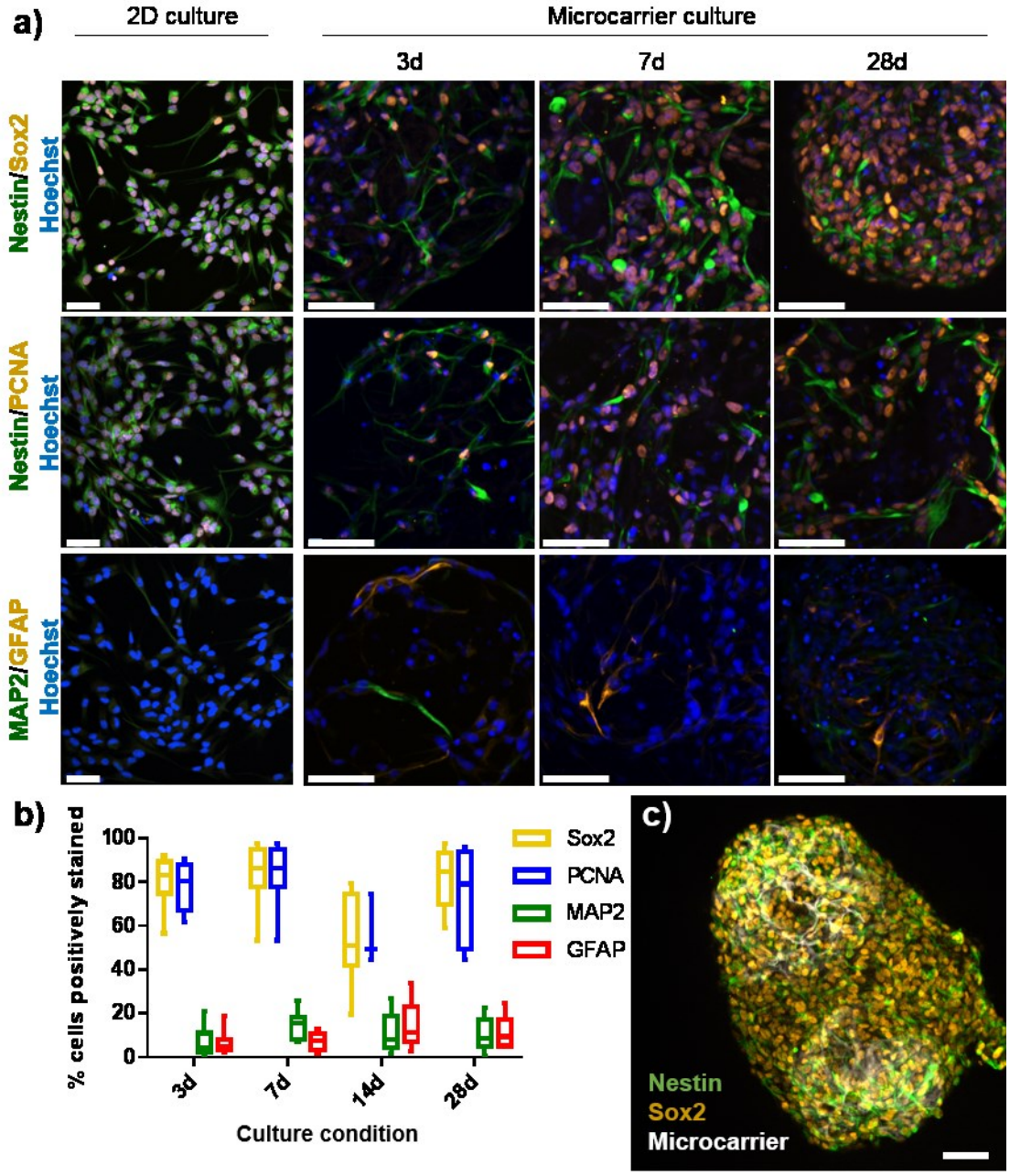

Figure 3. Immunocytochemistry of NPCs cultured on microcarriers under proliferative conditions shows that the majority of cells express nestin, Sox2 and PCNA. a) The top row indicate cells positive for nestin (green) and Sox2 (orange), the middle row for nestin (green) and PCNA (orange), the bottom row MAP2 (green), GFAP (orange) and counterstained with Hoechst 33342 (blue). b) Quantification of the above markers for NPCs cultured on microcarriers in proliferative conditions. The majority of cells are positive for cell division marker PCNA and are positive for Sox2 suggesting they remain in a multipotent state (n=3, 10 microcarriers per time point, total number of cells analyzed = 25,893, error bars represent ± standard deviation. c) A representative image of two microcarriers that have been bridged due to cell growth after 28 days in culture, showing that microcarriers contain an abundance of Sox2 and nestin positive cells. All scale bars mark 50 µm.

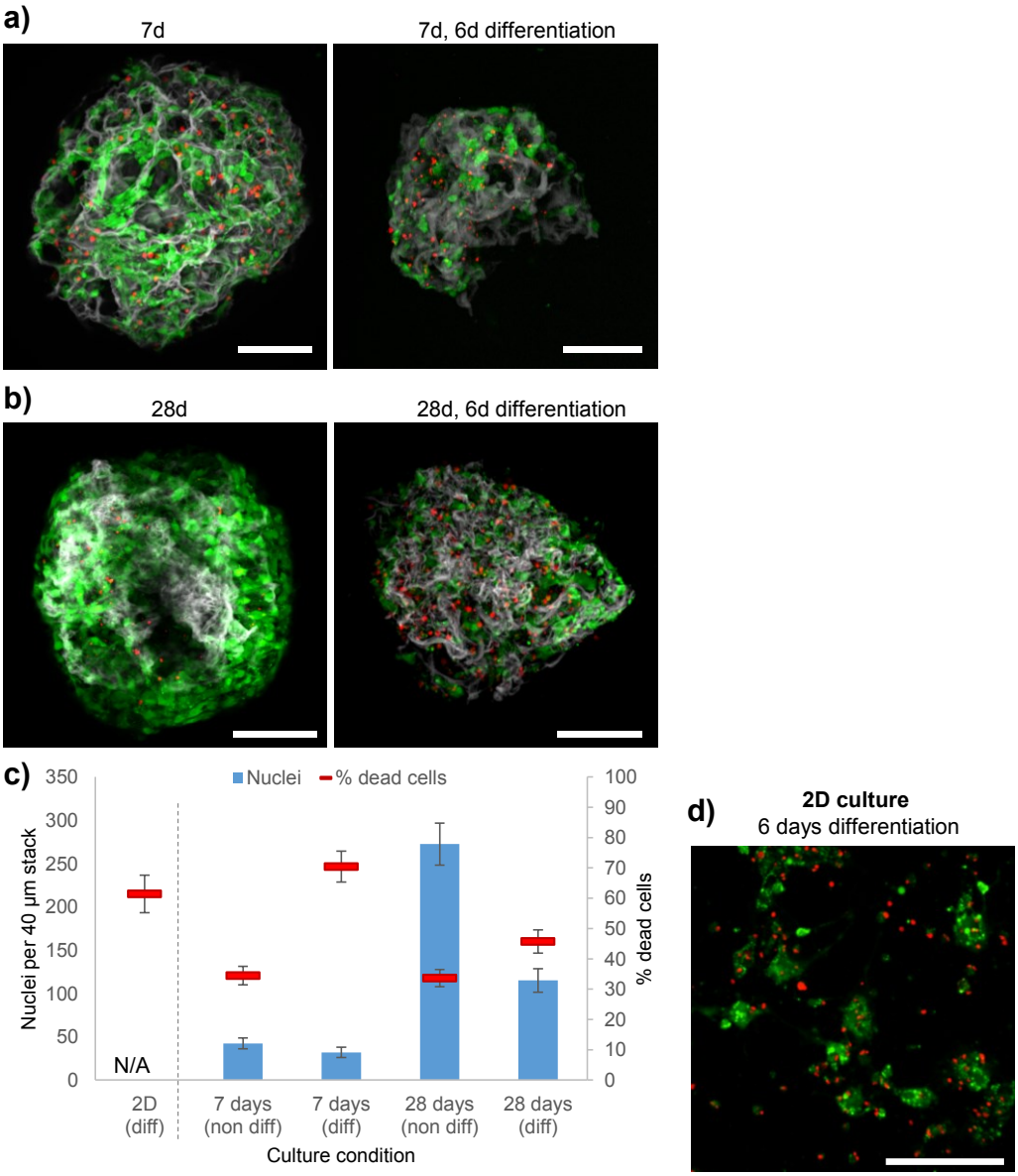

Figure 4. Live/dead analysis of the NPCs in differentiation conditions in comparison to proliferation conditions. Representative images of live cells (green) and dead cells (red) following NPC culture on microcarriers (white) for a) 7 days (left) or 7 days followed by 6 days of differentiation (right) or b) 28 days (left) or 28 days followed by 6 days of differentiation (right). c) Quantification of the number of nuclei and percentage of dead cells cultured in proliferation conditions (non diff) or differentiated (diff) for 6 days after different culture periods (until 80% confluency in the 2D case)(n=3, 30 microcarriers per time point, total number of cells analyzed = 14,688, error bars represent ± standard error of the mean). d) A representative image of NPCs grown in 2D culture and differentiated for 6 days. All scale bars mark 100 µm.

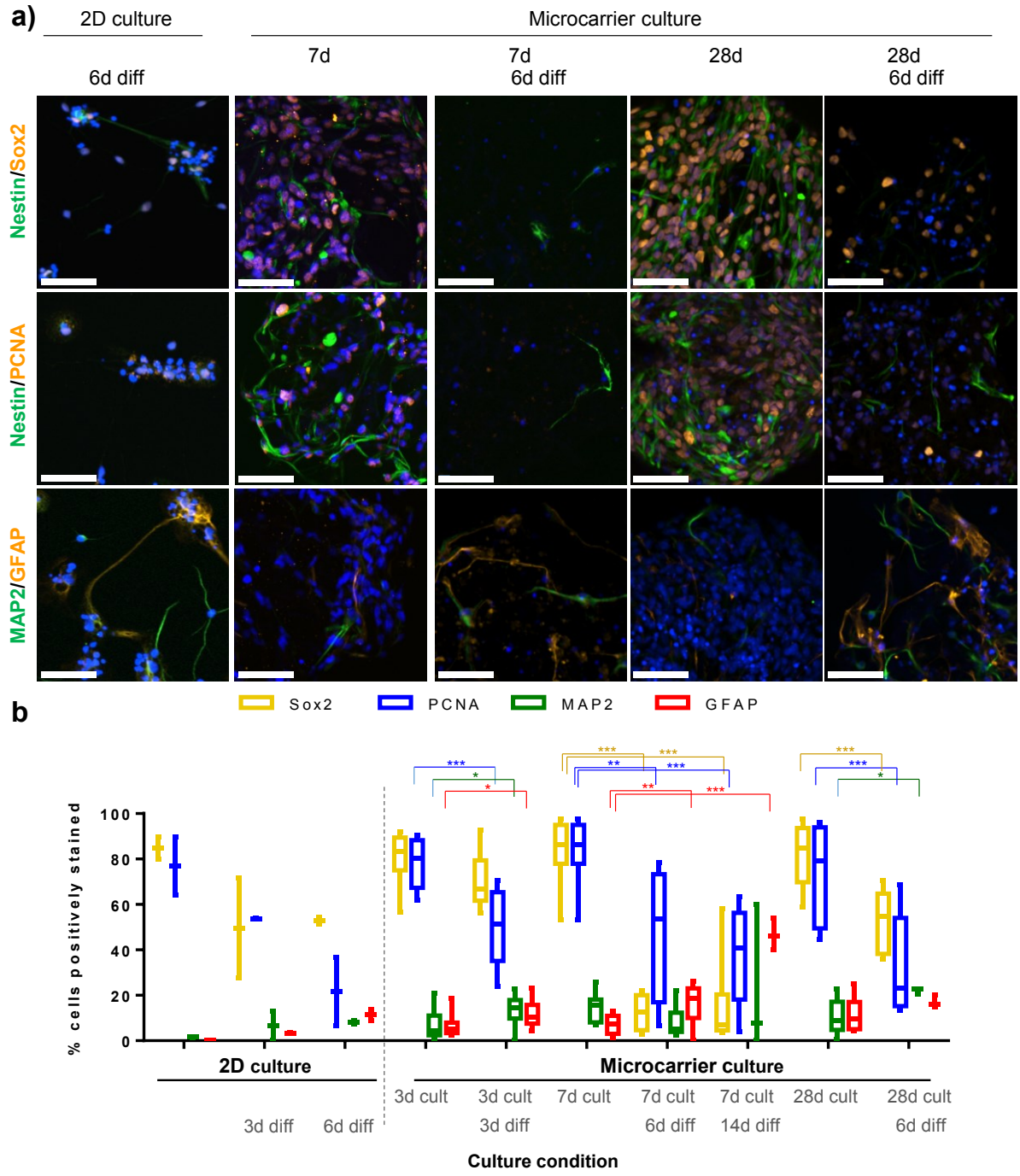

Figure 5. Immunocytochemistry analysis of NPC culture on microcarriers comparing proliferative conditions with differentiation conditions showing an increase in the percentage of differentiated cells upon mitogen withdrawal. a) Representative images of microcarrier cell culture performed for 7 and 28 days either with or without 6 subsequent days of differentiation. The top row indicate cells positive for nestin (green) and Sox2 (orange), the middle row for nestin (green) and PCNA (orange), the bottom row MAP2 (green), GFAP (orange) and counterstained with Hoechst 33342 (blue). For comparison, 2D NPC culture was grown to 80% confluency, differentiated for 6 days and stained. All scale bars mark 50 µm. b) Quantification of the immunocytochemical analysis of NPCs comparing 3, 7 and 28 days of culture on microcarriers either without differentiation or followed by various lengths of differentiation (diff). 2D culture, including 3 and 6 days of differentiation are included

(n=3, 10 microcarriers per time point, total number of cells analyzed = 28,164, error bars represent ± standard deviation). Asterisks represent a statistical significant difference between proliferation conditions and differentiation conditions for each time point (unpaired, two tailed t-test *$p<0.05$), **$p<0.01$) and ***$p<0.001$).

Supplementary Information

Macroporous heparin-based microcarriers allow long-term 3D culture and differentiation of neural precursor cells

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Materials and Methods

Cryogel microcarrier preparation and biofunctionalization

The cryogel microcarriers were prepared in a manner similar to that reported previously [1]. For preparation of the hydrogel precursor solution a molar ratio of four-arm amino-terminated PEG (10 kDa, JenKem Technology USA) to heparin (14 kDa, Sodium Salt, Porcine Intestinal Mucosa, Calbiochem, Merck) of 1:1 was used. A molar ratio of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich) to N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, Sigma) of 2:1 was used. 1% of the heparin used in the cryogel microcarrier preparation was Alexa 647 labelled (prepared from Alexa 647, Invitrogen as reported previously [1]). All components were dissolved in deionized, decarbonized water (MilliQ) by vortexing and ultrasonication and were kept on ice afterwards. Activation of carboxylic acid groups of heparin was performed by mixing the EDC, sulfo-NHS and heparin solutions and leaving them on ice for 15 minutes. Then the PEG solution was added and 600 µL of this final precursor solution were immediately injected into a mixture of 9 mL toluene and 3 mL of a Synperonic PEP105 (Sigma-Aldrich) in toluene solution (c = 1 mg/mL, as a stabilizing agent). The resulting emulsion was stirred at 700 rpm for 10 minutes at room temperature for droplet formation to occur, before another 3 mL of Synperonic PEP105 in toluene solution (1 mg/mL) were added to the stirring solution to avoid droplet agglomeration. Subsequently, the flask containing the emulsion was immersed into a -80 °C ethanol bath for 2 hours. The resulting microcarriers were kept at -20 °C over-night and subsequently lyophilized for at least 24 h, before they were dispersed in ethanol and afterwards washed several times with MilliQ water.

The microcarriers were used as such (without RGD) or they were functionalized with a cyclic peptide sequence containing the RGD motif (cyclo(Arg-Gly-Asp-D-Tyr-Lys) PCI-3662-PI, (Peptides International). For RDG-functionalization 5 mg of dry microcarriers were activated by sterile filtered EDC (28.8 mg) and sulfo-NHS (16.3 mg) (molar ratio of 2:1) solution in 1.5 mL phosphate buffer (pH=5) for 45 min at 4 °C while shaking at 400 rpm. The buffer was exchanged to borate buffer (pH=8) before adding a 200 µg/mL solution of the cyclic peptide dissolved in borate buffer. Incubation lasted for 2 h at room temperature shaking at 400 rpm. Functionalized microcarriers were washed with MilliQ water three times, frozen in liquid nitrogen and lyophilized.

Microcarrier characterization

The dried microcarriers were attached to the sample holder via carbon adhesive, and sputtered with gold for 60 s at 40 mA (SCD 050 Sputter Coater, Balzers). These samples were then imaged using a XL30 ESEM-FEG scanning electron microscope (Philips) using the secondary electron detector and acceleration voltages of 3.0 - 5.0 kV. A Dragonfly spinning disc confocal laser microscope (SPCLM) (Andor Technology Ltd, Belfast, Ireland) mounted on a Nikon Ti-E inverted microscope was used to visualize the Atto 647 labelled microcarriers in the hydrated state (phosphate-buffered saline solution (PBS)), with a 637 nm laser diode and images were taken with a 10x magnification objective (CFI Plan Apo Lambda, Nikon). Microcarrier diameter and pore size were determined using ImageJ software (NIH) as described previously [1, 2].

Neural precursor cell (NPC) culture

Hippocampal neural precursor cells (NPCs) extracted from the dentate gyri of 6-8 week old C57BL/6 mice (as previously reported [3]) were cultivated for up to 7 passages on poly-D-lysine and laminin coated flasks in neurobasal medium (NB medium, Gibco) supplemented with B27 (Gibco) (NB + B27 medium) at 37 °C in 5% CO_2 at 90% humidity [4]. In non-differentiating conditions human epidermal growth factor (EGF, Peprotech) (10 ng/mL) and fibroblast growth factor (bFGF, Peprotech) (10 ng/mL) were added to the medium. The medium was changed every other day.

2D NPC culture - For 2D NPC experiments 40,000 cells were seeded on laminin and poly-D-lysine coated 12 mm glass cover slips and allowed to proliferate until 80% confluency was reached. Part of this culture was fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes and stored in Dulbecco´s phosphate-buffered saline supplemented with calcium and magnesium (DPBS) at 4°C until stained. In parallel, the non-fixed cells in 2D culture were differentiated via the withdrawal of growth factors. NPCs were differentiated for 3 and 6 days and subsequently fixed with 4% PFA in PBS for 10 minutes and stored in DPBS at 4°C until staining was carried out.

3D NPC culture on the microcarriers - NPCs were removed from the 2D culture flask by incubation with Accutase (Sigma-Aldrich) for 3 min at 37°C. 500,000 cells were pipetted to 1.5 mg of dry RGD-functionalized microcarriers in a centrifuge tube and topped up with 100 µL of NB + B27 medium. The cell loaded microcarriers were incubated for 1 h at 37 °C before being transferred to a 12-well plate for non-adherent culture (static culture) or a Sigmacote (Sigma-Aldrich) treated spinner flask (dynamic culture). Wells contained 1 mL of medium or spinner flasks (GPE Scientific Ltd – standard 25 mL flat bottom flask) contained 25 mL of medium (medium = NB + B27 media with growth factors EGF and bFGF as described above). A spin speed of 80 rpm was used, controlled by a Cimarec i Poly 15 stir plate (ThermoFisher). For proliferation conditions, half of the medium was changed every other day. Analysis was performed after 3, 7, 14 and 28 days. Differentiation of the 3D culture was induced by withdrawal of growth factors and samples were analyzed after a further 1, 3 , 6 and 14 days. For 3D culture experiments on Cultispher S (Sigma-Aldrich), the Cultisphers were first sterilized and dried according to the manufacturer's protocol, and used in an identical manner to the cryogel microcarriers.

Immunocytochemistry staining

2D NPC cultures and cells on microcarriers were fixed with 4% PFA in PBS, permeabilized with 0.1% Triton-X100 (Sigma) for 15 minutes, blocked with 10% donkey serum for 30 minutes. Subsequently, cells were incubated with either rabbit anti-Sox2 (Millipore, AB5603, 1:400) and mouse anti-nestin (BD Biosciences, 611658, 1:250), rabbit anti-PCNA (abcam, ab18197, 1:1000) and mouse anti-nestin (BD Biosciences, 611658, 1:250) or rabbit anti-GFAP (Dako, Z0334, 1:250) and mouse anti-Map2 (Sigma, M1406in, 1:1000) in blocking solution at 4 °C over-night. After washing with 1X DPBS three times, secondary antibody incubation was performed with donkey anti-rabbit Alexa Fluor568 IgG (H+L) (Life technologies, A10042, 1:500) and donkey anti-mouse Alexa Fluor488 IgG (H+L) (Dianova, 715-545-151, 1:500) in blocking solution for 3 hours at room temperature in the dark. Unbound secondary antibody was washed away with 1X DPBS. Thereafter, cells were counterstained with Hoechst 33342 nuclear dye (Molecular Probes, Invitrogen, 1:1000 in DPBS) for 10 min and again washed with 1X DPBS.

Live-dead staining

To determine the percentage of dead cells, a live-dead solution (calcein AM Solution (PromoKine) and propidium iodide (Sigma)) was added to cells cultured on microcarriers in NB + B27 medium and incubated for 20 min at 37 °C. Cells were counterstained with Hoechst 33342 (1:1000 in DPBS) for 10 min and washed with NB medium once. For the cell harvesting experiment, microcarriers with cells

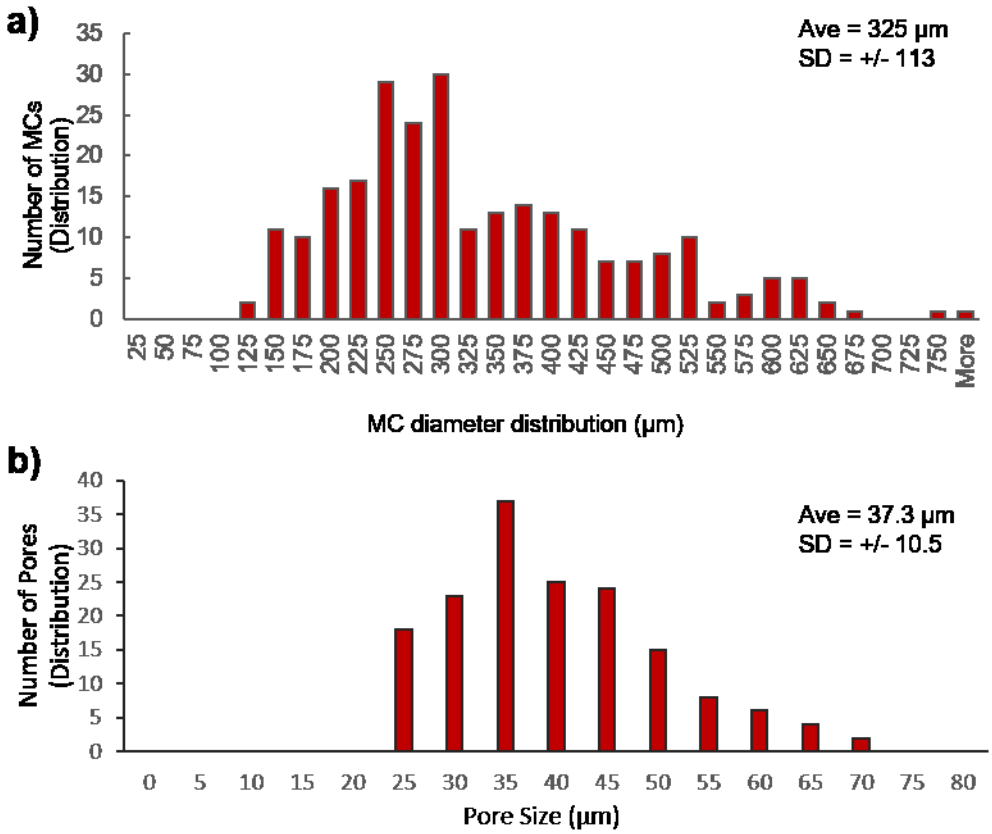
were pelleted at 50g for 1 min, followed by incubation in 500µl accumax or accutase for 5 min. The cells were washed once in PBS, and then resuspended in PBS containing trypan blue and analyzed using a Countess 2 cell counter (Life Technologies). The number and percentage of viable cells was counted. The microcarriers were then analyzed separately by qualitative confocal microscopy.

Microscopy analysis

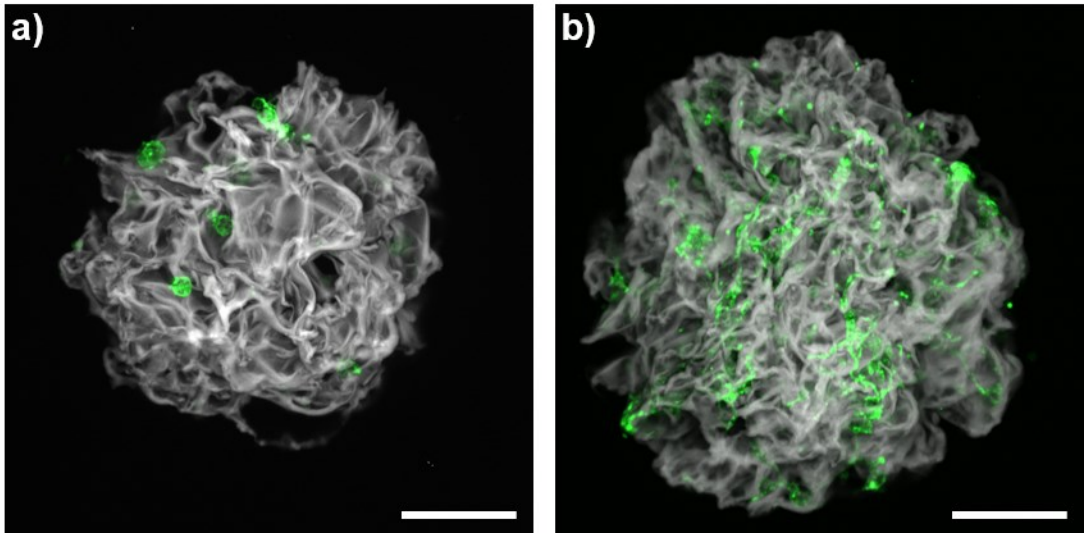
Immediately after live-dead staining cells were imaged with a Dragonfly spinning disc confocal laser microscope at 20x magnification. Per culture condition 15 z-stacks of 40 µm depth with a 1 µm step size were taken for quantitative analysis. For qualitative images, 40 µm and 80 µm z-stacks with step sizes of 0.5 µm were acquired. For each microcarrier a single image of the cross section was taken to determine its diameter. Analysis of pictures was performed using ImageJ (NIH). For quantitative analysis images were processed by subtracting the background with a rolling ball radius of 5 pixels. Cell nuclei were counted automatically by 3D objects counter v2.0 with a size filter set to 100 minimum. Mean values for the total amount of cells, the amount of dead cells, the number of Sox2 and PCNA positive cells were determined. Map2 and GFAP positive cells were counted manually. Ratios of amount of dead cells, Sox2, PCNA, MAP2 or GFAP positive cells to total amount of cells per microcarrier were calculated and plotted. For qualitative analysis, the microscopy images were processed with Imaris software (Bitplane).

Statistical analysis

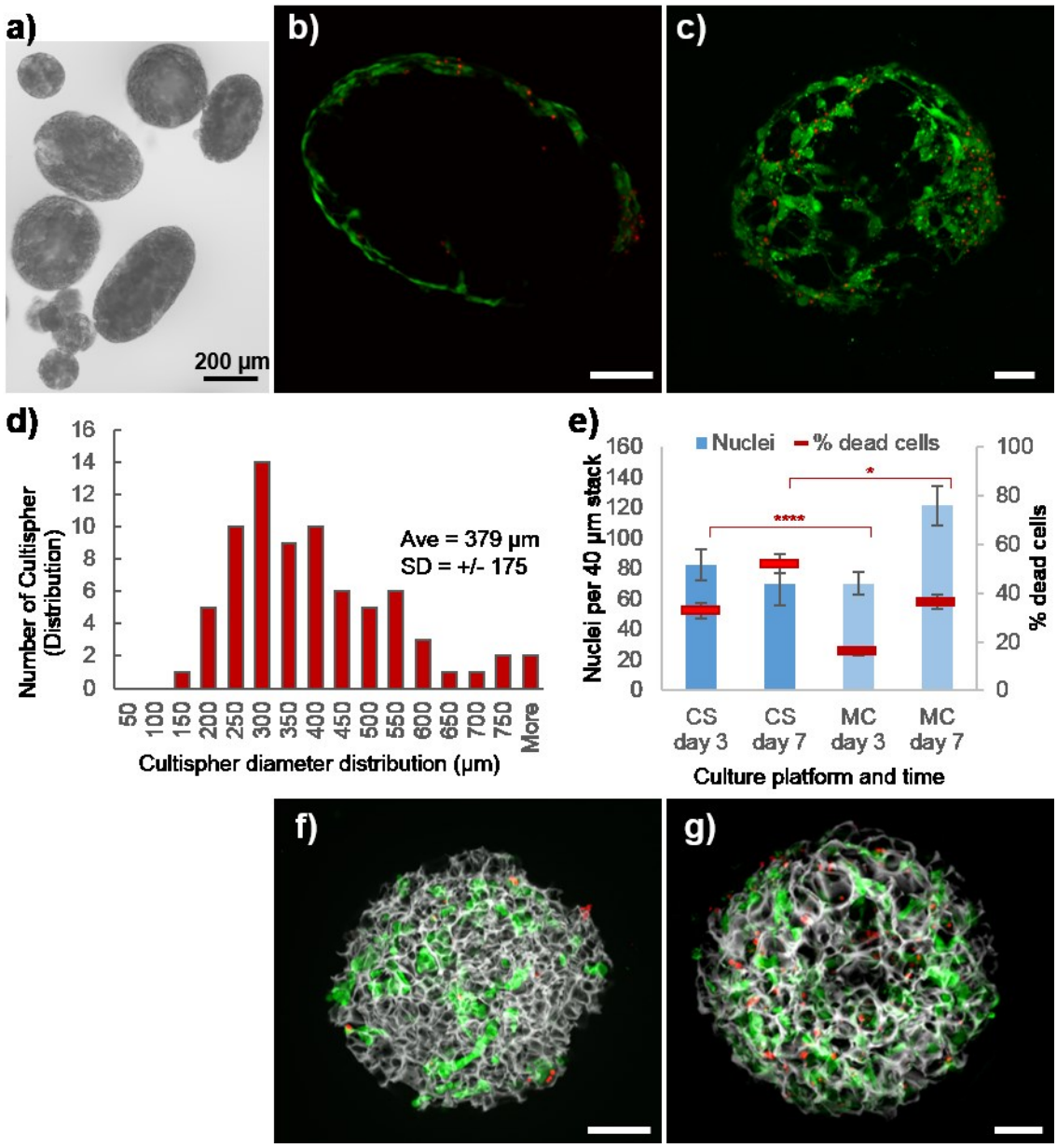
Microsoft Excel was used to determine the R2 values and Graph Pad Prism software was used to perform the unpaired t-test analysis comparing differentiation conditions to the equivalent proliferation conditions. The same test was used for the comparison between microcarrier and Cultispher culture. Three replicates were carried out for each study and the total number of microcarriers and the total number of cells analyzed for each condition is indicated in the figure legends.



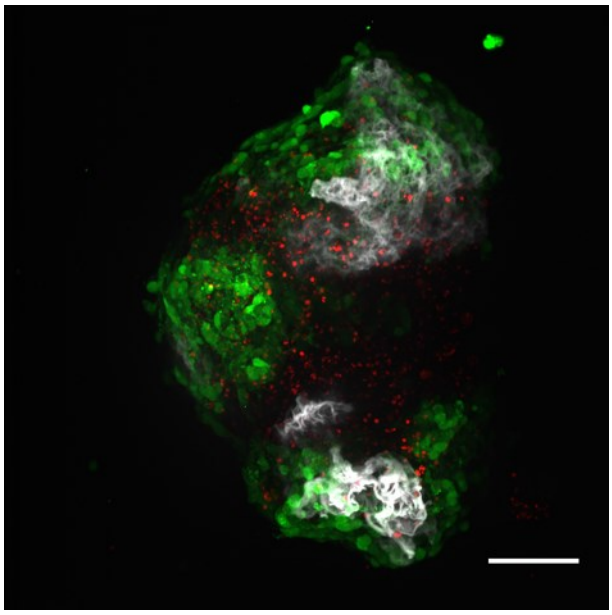
Supplementary Figure S1. Size distribution analysis of the microcarriers in the hydrated state (upper panel), and subsequent pore size distribution (lower panel), with averages (Ave) and standard deviations (SD) indicated.



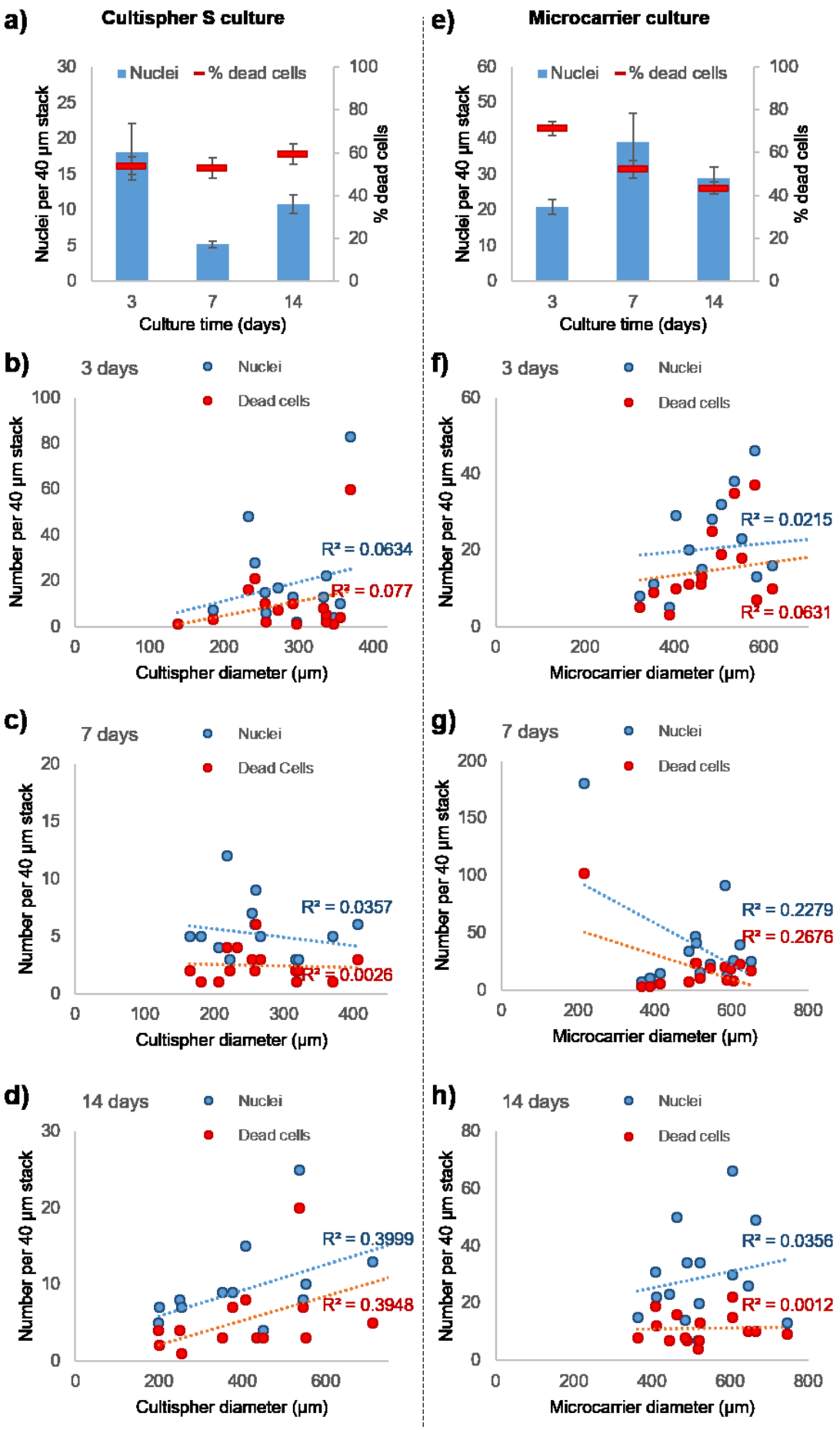
Supplementary Figure S2. Functionalization of the microcarriers with the RGD motif is required for NPC adhesion. Qualitative analysis of cell attachment and spreading of NPCs to the microcarriers after three days in culture is shown via Phalloidin-488 stained NPCs (green) on Alexa 647 labelled microcarriers (white) either without RGD functionalization (a) or with the RGD motif (b). Scale bars represent 100 µm.



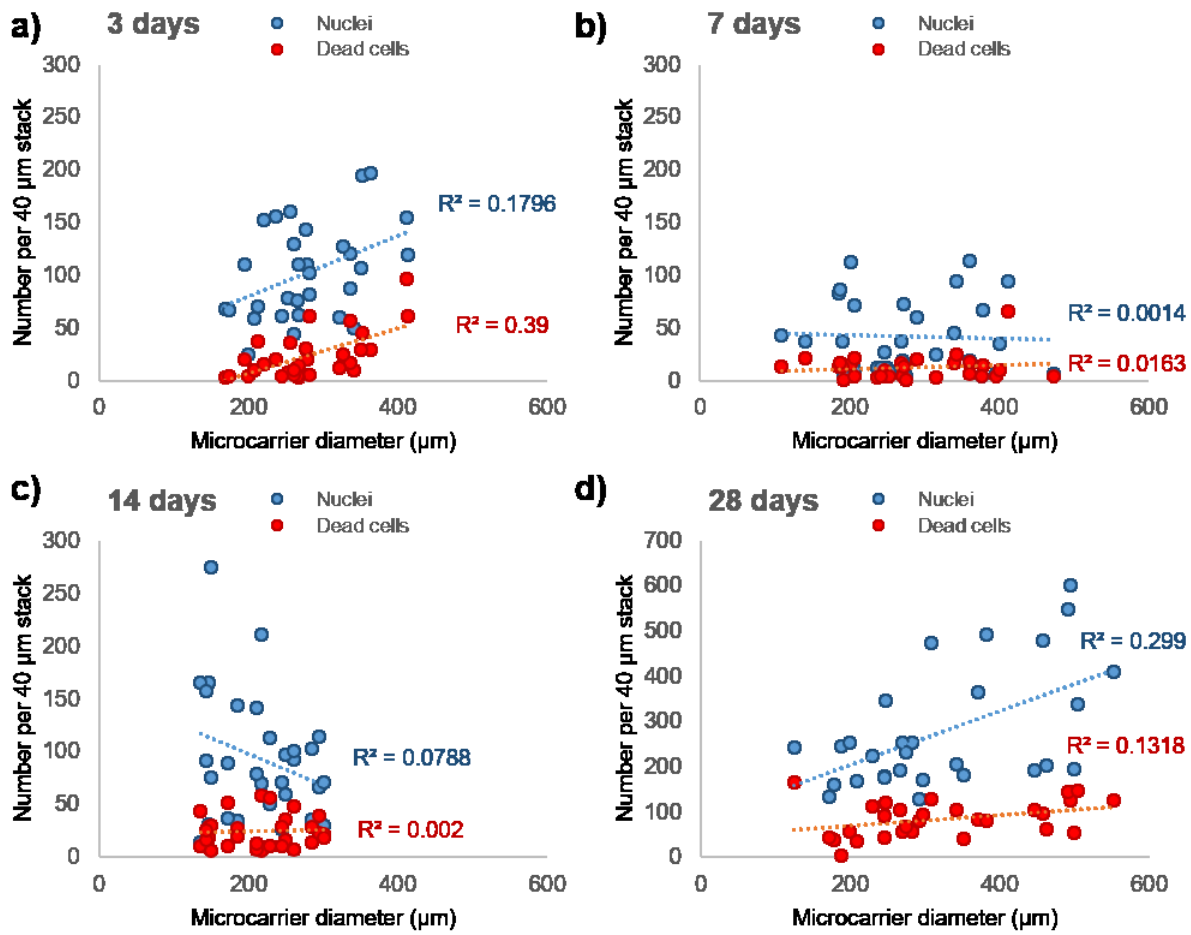
Supplementary Figure S3. a) Representative brightfield image of the Cultispheres resuspended in phosphate buffered saline as per the manufacturers' protocol. Representative images of live cells (green) and dead cells (red) following NPC culture on Cultispheres for b) 3 days or c) 7 days. d) Quantification of the size distribution of the Cultispheres (total of 75 measured). e) Quantification of the number of nuclei and percentage of dead cells cultured in proliferation conditions on either the Cultispheres (CS) or microcarriers (MC) contained within a 40 µm z plane (n=3, 15 MC or CS per time point, total number of cells analyzed = 5,174, error bars represent ± standard error of the mean). Asterisks represent a statistical significant difference between the CS and MC in terms of percentage of dead cells for each time point (no statistically significant difference between the number of nuclei on CS and MC per time point was observed)(unpaired, two tailed t-test *p<0.05, ****p<0.0001). Representative live/dead images of NPCs cultured on microcarriers (white) after f) 3 days and g) 7 days in culture. All scale bars mark 100 µm.



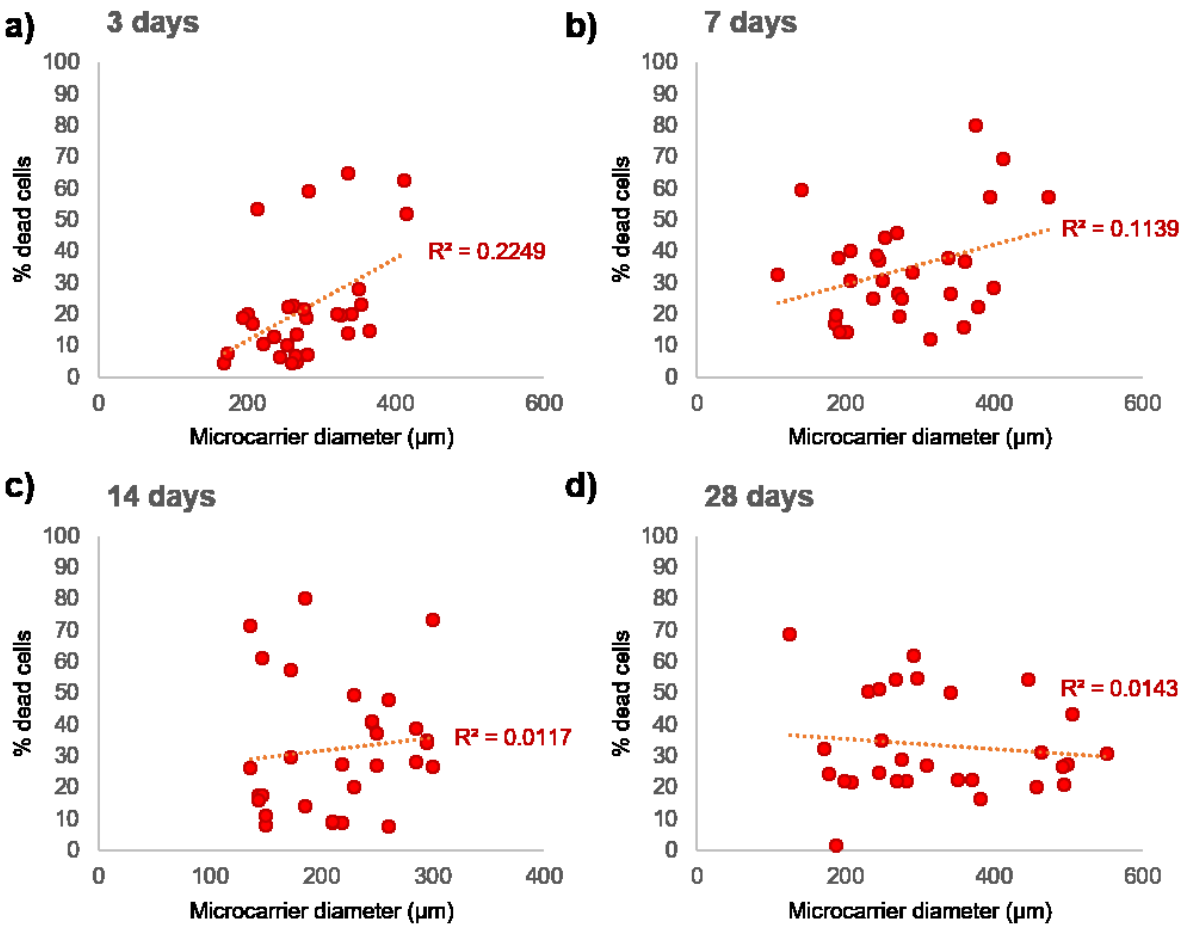
Supplementary Figure S4. Representative image of live cells (green) and dead cells (red) after 28 days of NPC culture on microcarriers (white). This image shows how the microcarriers have clustered as NPC connect them together. This was not observed in any of the previous time points analyzed. Scale bar marks 100 μm.


a)
Cultispher S culture
Nuclei
% dead cells
Nuclei per 40 µm stack
% dead cells
Culture time (days)
e)
Microcarrier culture
b)
3 days
Dead cells
Number per 40 µm stack
R² = 0.0634
R² = 0.077
Cultispher diameter (µm)
f)
R² = 0.0215
R² = 0.0631
Microcarrier diameter (µm)
c)
7 days
Dead Cells
R² = 0.0357
R² = 0.0026
g)
R² = 0.2279
R² = 0.2676
d)
14 days
R² = 0.3999
R² = 0.3948
h)
R² = 0.0356
R² = 0.0012

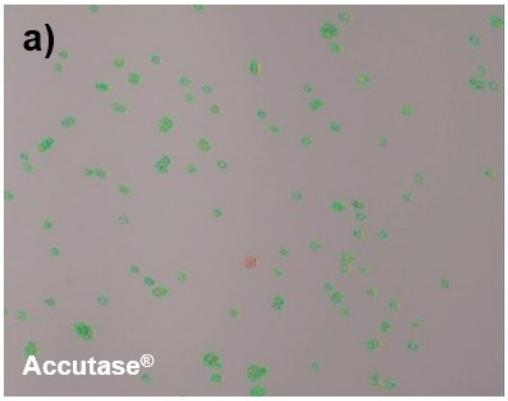
Supplementary Figure S5. Dynamic culture of NPCs on either Cultisphers or microcarriers in spinner flasks was not successful. Cells were cultured on microcarriers as per the static culture for 1 hour then transferred to spinner flasks (GPE Scientific Ltd) in a total volume of 25 mL with a spin speed of 80 rpm (controlled by a Cimarec i Poly 15 stir plate (ThermoFisher)). Quantification of the number of nuclei and the number of dead cells per 40 µm z-range of either Cultisphers (a) or microcarriers (e)(15 Cultisphers or 15 microcarriers per time point, error bars represent ± standard error of the mean). Analysis of the number of nuclei and dead cells, plotted in comparison to the Cultispher diameter (b-d) or microcarrier diameter (f-h) for culture in proliferative conditions for 3 days (b and f), 7 days (c and g) or 14 days (d and h).

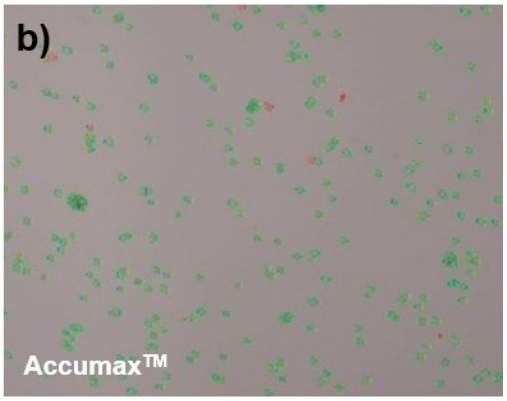


Supplementary Figure S6. Microcarrier diameter does not affect the number of dead cells on the microcarriers. Quantification of the number of nuclei and the number of dead cells per 40 µm z-range plotted in comparison to the microcarrier diameter for culture in proliferative conditions for a) 3 days, b) 7 days, c) 14 days and d) 28 days.



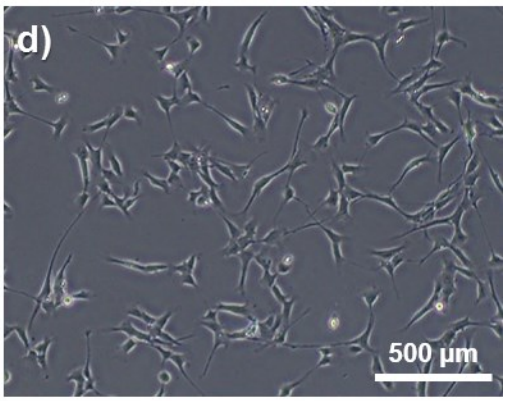
Supplementary Figure S7. Microcarrier diameter does not affect the number of dead cells on the microcarriers. Quantification of the percentage of dead cells per 40 µm z-range plotted in comparison to the microcarrier diameter for culture in proliferative conditions for a) 3 days, b) 7 days, c) 14 days and d) 28 days.

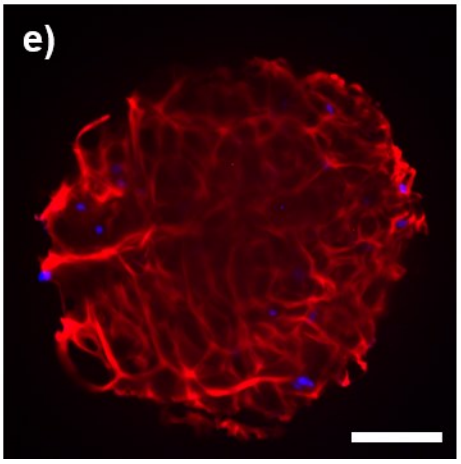


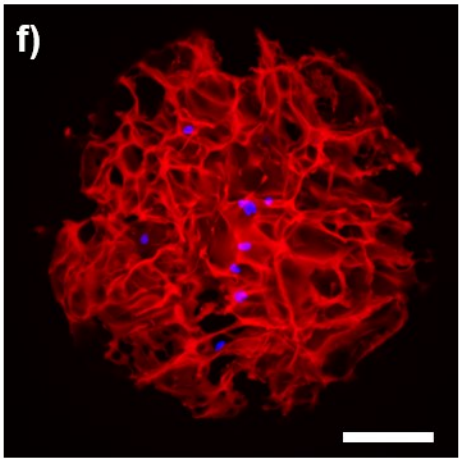


c)

	Accutase	Accumax
% Recovered	30.0	60.0
% Live (trypan blue excluded)	97.4	95.0
% Dead (trypan blue)	2.6	5.0







Supplementary Figure S8. Representative images taken via a Countess 2 (Life Technologies) showing live cells (green) and dead cells (red) during quantification of the number of cells harvested from the microcarriers (three days in culture – proliferation conditions), after removal via a) accutase® or b) accumax™. c) Quantification of the cell viability via trypan blue exclusion quantified using the Countess 2. d) A representative image of the cells 12 hours post re-seeding in a PDL/laminin coated flask showing normal NPC morphology. Representative images of the nuclear staining (Hoechst 33342 – blue) after e) accutase or f) accumax harvesting of the cells, showing that the microcarriers (red) are largely devoid of cells, though a few have remained post harvesting.

References

[1] Newland B, Welzel PB, Newland H, Renneberg C, Kolar P, Tsurkan M, et al. Tackling cell transplantation anoikis: An injectable, shape memory cryogel microcarrier platform material for stem cell and neuronal cell growth. Small. 2015;11:5047-53.
[2] Eigel D, Zoupi L, Sekizar S, Welzel PB, Werner C, Williams A, et al. Cryogel scaffolds for regionally constrained delivery of lysophosphatidylcholine to central nervous system slice cultures: A model of focal demyelination for multiple sclerosis research. Acta Biomaterialia. 2019.
[3] Walker TL, Kempermann G. One mouse, two cultures: Isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. JoVE. 2014:e51225.
[4] Ehret F, Vogler S, Kempermann G. A co-culture model of the hippocampal neurogenic niche reveals differential effects of astrocytes, endothelial cells and pericytes on proliferation and differentiation of adult murine precursor cells. Stem Cell Research. 2015;15:514-21.

Figures

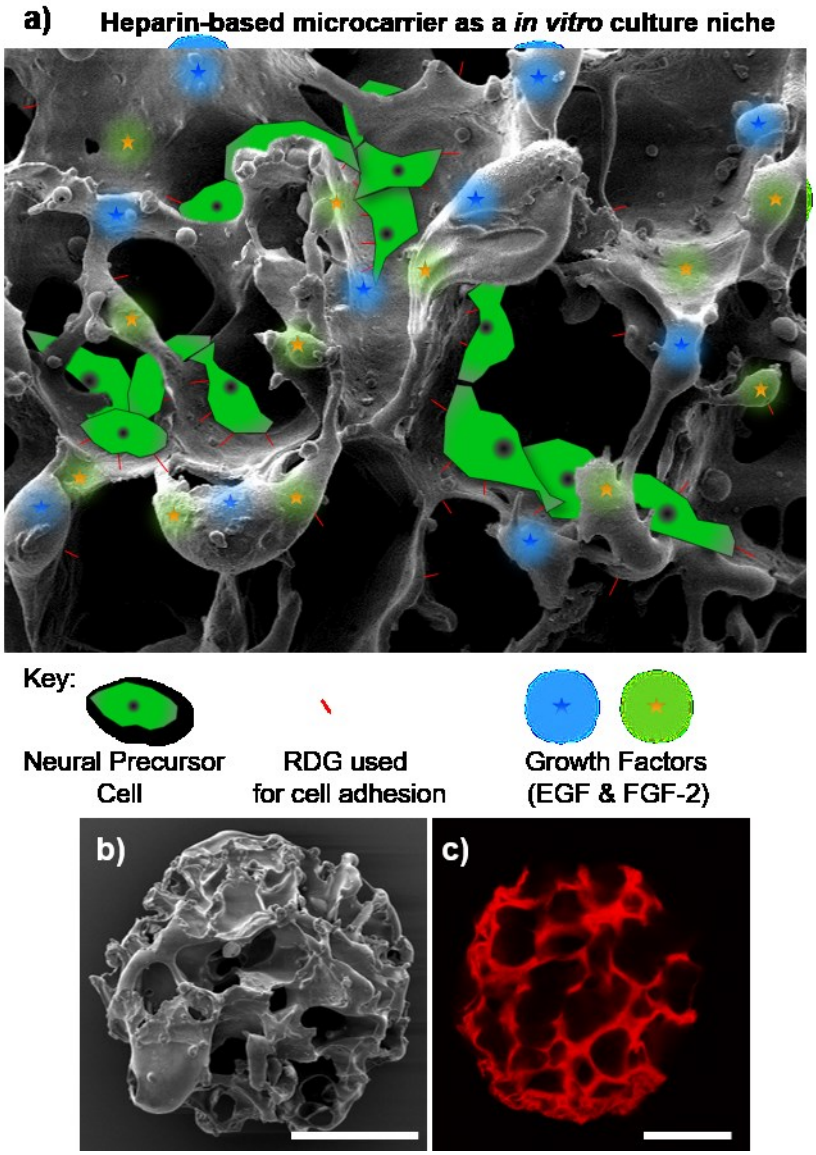


Figure 1. **The microcarriers are synthesized via a cryogelation process, which leaves them with a macroporous structure.** a) Schematic depiction of NPCs cultured on the microcarriers comprised of poly(ethylene glycol), heparin and the RGD peptide. b) Scanning electron microscope image of a microcarrier under vacuum. b) confocal laser scanning microscopy image of a hydrated microcarrier in cell culture medium (scale bars represent 50 μm).

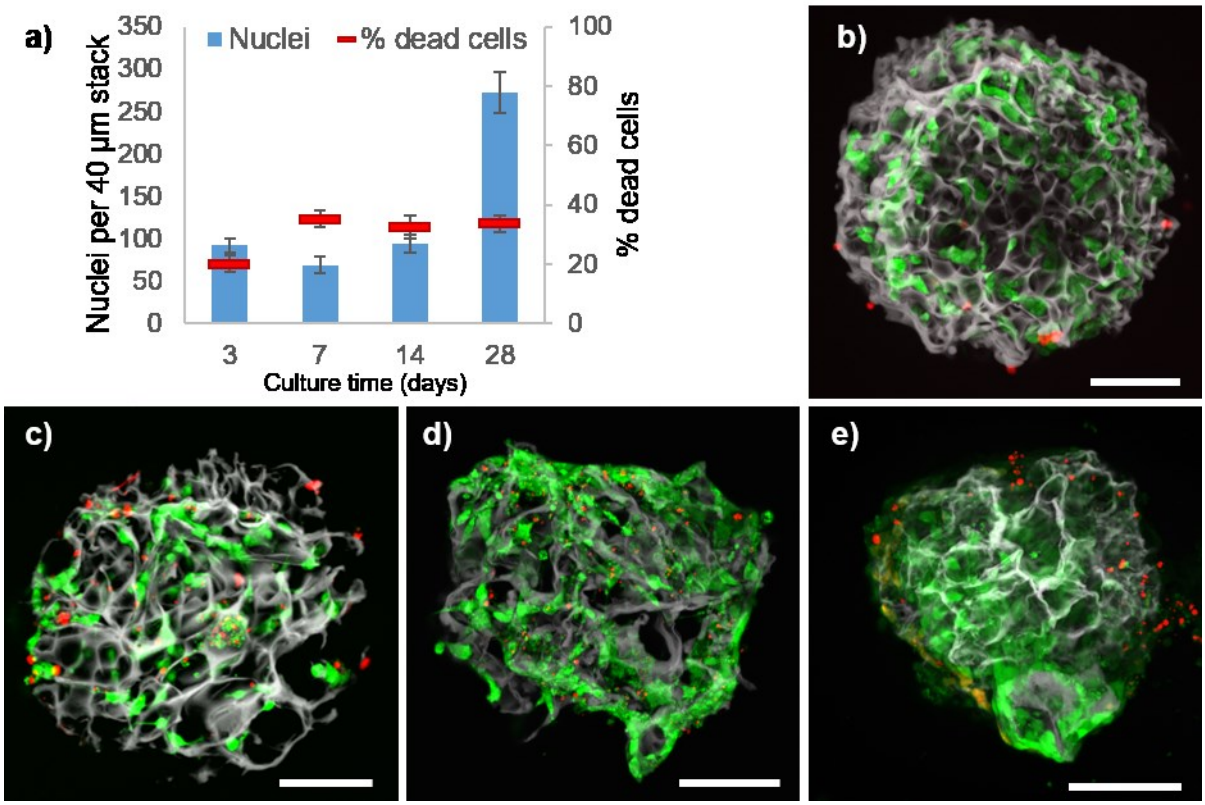


Figure 2: Live/Dead fluorescence staining of NPC culture on microcarriers (MC) in proliferative conditions for 3, 7, 14 and 28 days. a) Quantification of the number of nuclei in a 40 µm z plane of the microcarrier with corresponding percentage of dead cells (n=3, 30 microcarriers per time point, total number of cells analyzed = 15,340, error bars represent ± standard error of the mean). Representative images of calcein positive live cells (green) and propidium iodide positive dead cells (red) after b) 3 days, c) 7 days, d) 14 days and e) 28 days of culture on the microcarriers (white). Scale bars represent 100 µm.

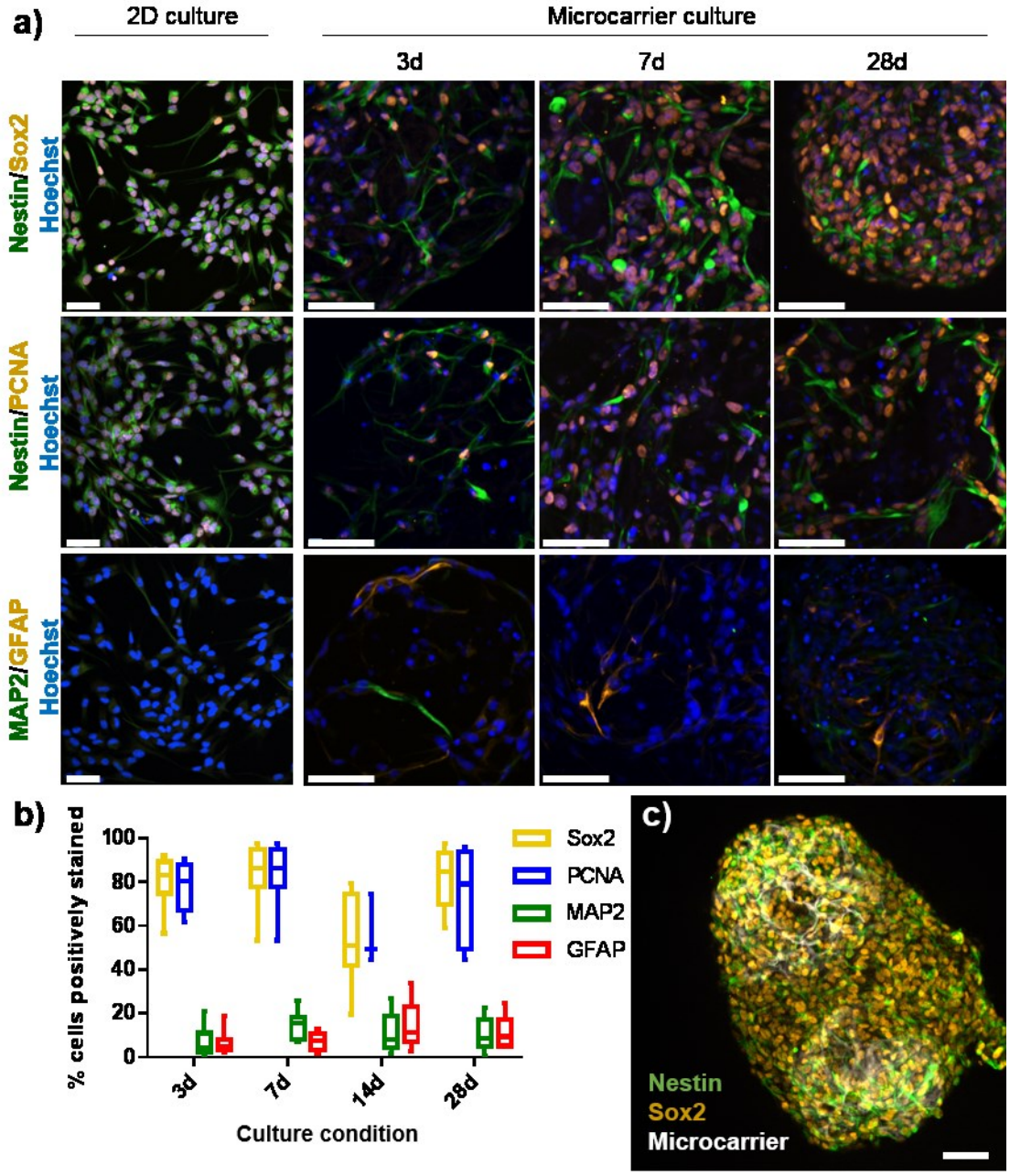


Figure 3. **Immunocytochemistry of NPCs cultured on microcarriers under proliferative conditions shows that the majority of cells express nestin, Sox2 and PCNA.** a) The top row indicate cells positive for nestin (green) and Sox2 (orange), the middle row for nestin (green) and PCNA (orange), the bottom row MAP2 (green), GFAP (orange) and counterstained with Hoechst 33342 (blue). b) Quantification of the above markers for NPCs cultured on microcarriers in proliferative conditions. The majority of cells are positive for cell division marker PCNA and are positive for Sox2 suggesting they remain in a multipotent state (n=3, 10 microcarriers per time point, total number of cells analyzed = 25,893, error bars represent ± standard deviation. c) A representative image of two microcarriers that have been bridged due to cell growth after 28 days in culture, showing that microcarriers contain an abundance of Sox2 and nestin positive cells. All scale bars mark 50 µm.

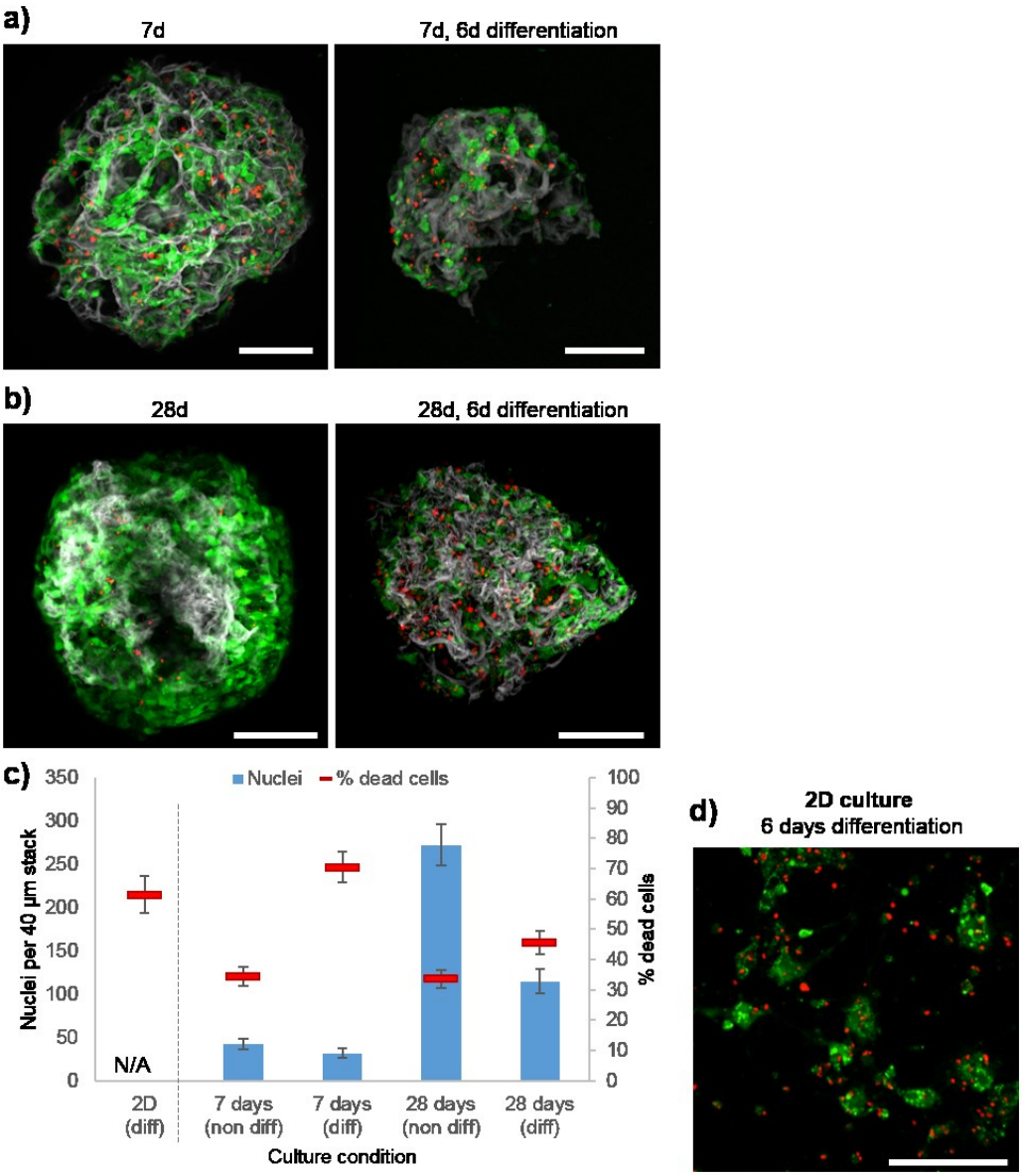


Figure 4. **Live/dead analysis of the NPCs in differentiation conditions in comparison to proliferation conditions.** Representative images of live cells (green) and dead cells (red) following NPC culture on microcarriers (white) for a) 7 days (left) or 7 days followed by 6 days of differentiation (right) or b) 28 days (left) or 28 days followed by 6 days of differentiation (right). c) Quantification of the number of nuclei and percentage of dead cells cultured in proliferation conditions (non diff) or differentiated (diff) for 6 days after different culture periods (until 80% confluency in the 2D case)(n=3, 30 microcarriers per time point, total number of cells analyzed = 14,688, error bars represent ± standard error of the mean). d) A representative image of NPCs grown in 2D culture and differentiated for 6 days. All scale bars mark 100 µm.

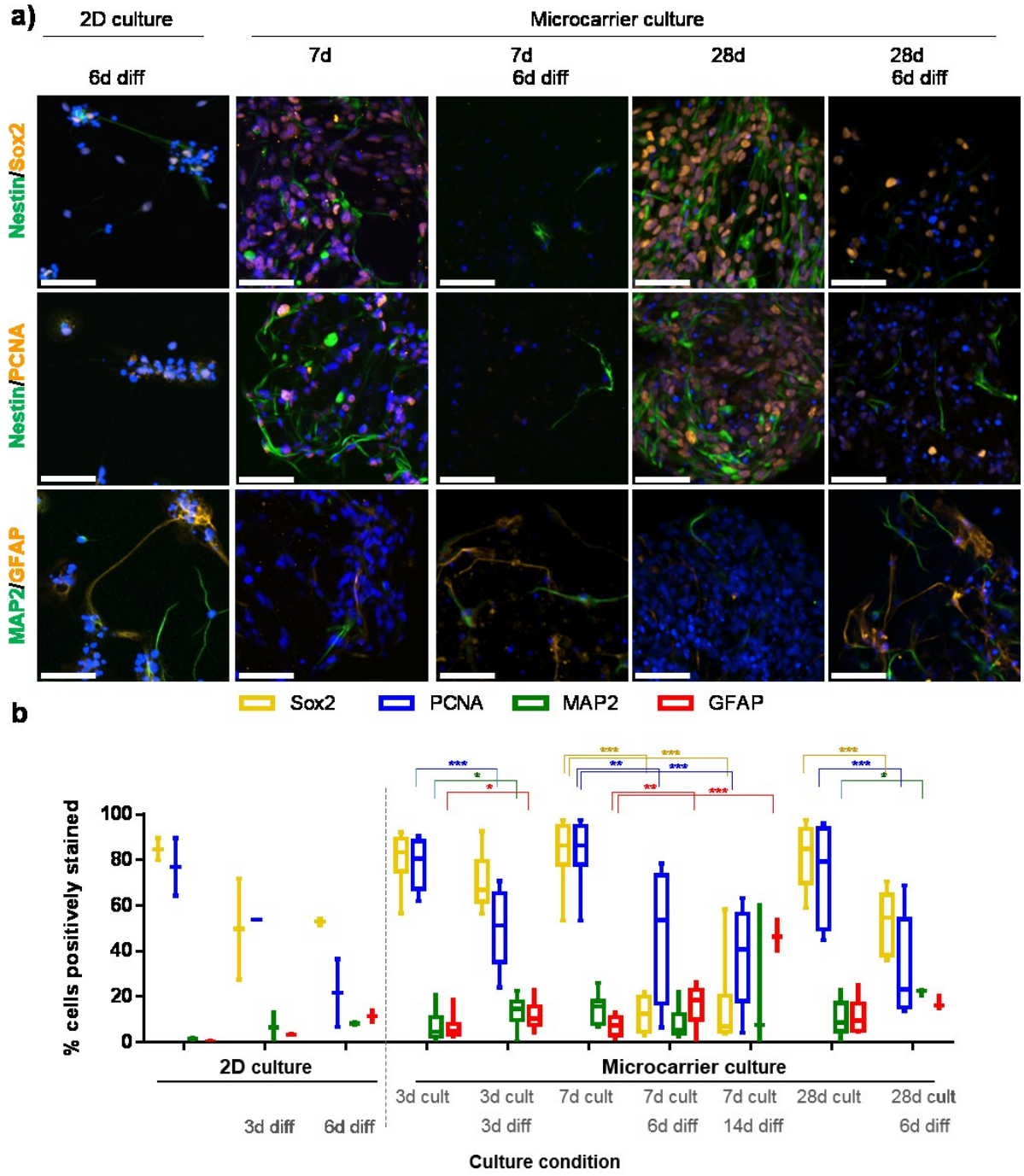


Figure 5. **Immunocytochemistry analysis of NPC culture on microcarriers comparing proliferative conditions with differentiation conditions showing an increase in the percentage of differentiated cells upon mitogen withdrawal.** a) Representative images of microcarrier cell culture performed for 7 and 28 days either with or without 6 subsequent days of differentiation. The top row indicate cells positive for nestin (green) and Sox2 (orange), the middle row for nestin (green) and PCNA (orange), the bottom row MAP2 (green), GFAP (orange) and counterstained with Hoechst 33342 (blue). For comparison, 2D NPC culture was grown to 80% confluency, differentiated for 6 days and stained. All scale bars mark 50 µm. b) Quantification of the immunocytochemical analysis of NPCs comparing 3, 7 and 28 days of culture on microcarriers either without differentiation or followed by various lengths of differentiation (diff). 2D culture, including 3 and 6 days of differentiation are included

(n=3, 10 microcarriers per time point, total number of cells analyzed = 28,164, error bars represent ± standard deviation). Asterisks represent a statistical significant difference between proliferation conditions and differentiation conditions for each time point (unpaired, two tailed t-test *$p<0.05$), **$p<0.01$) and ***$p<0.001$).