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Figure 11. Three-dimensional migration assay. Representative images of migrated neonatal olfactory bulb ensheathing cells (NOBECs) 0 (a), and 72 h (b) after incorporation in A/GL-GP (agar and gelatin cross-linked with genipin) hydrogel. Cells were stained with cell tracker green (CTG) and visualized in green; biomaterial autofluorescence is visualized in red. Reprinted from: Tonda-Turo et al.[173]

Cell Encapsulation of co-cultures offers to study cell–cell and natural ecosystem interactions.[68, 174] The immediately advantage of this technique is a more detailed representation of physiological environment compared with monoculture, by the control of several factors like, type of medium, cell concentration, time scale, and the addition of degree of contact between cell populations. The degree of separation can be achieved through semipermeable membranes, gels, microfluidic devices, and others.[68, 69, 175] The way in which the cell-culture degree of separation is arranged defines the experimental set-up, for this case 3D arrangements with scaffolds and hydrogels will be highlighted. Challenges that bound this technique is the high level of complexity mainly because of the multiple cell interactions generating unpredictability and instability, which leads to another challenge of poor knowledge of data acquisition tools since it involves multiple interactions and even ones that are unknown.[68, 174, 175]

Co-culture experimental setup depends upon the separation and suspension techniques or materials in which the cell populations will be exposed.[68] It is important to highlight that the applied technique defines whether it will be a two or 3D structure. An example of a co-culture technique that generates a 3D structure is spheroidal co-culture, also known as MCS, which are developed by a number of techniques, including hanging drops culture, single-cell culture on non-adhesive surface, micromolding techniques, spinner flask culture, rotary cell systems, porous 3D scaffolds, and centrifugation pellet culture.[175] The co-culture spheroids were suspended in a 3D collagen matrix and together represented a 3D structure for peripheral nerve regeneration.[69, 176] The co-cultured consisted of SCs (500 cells/spheroid) and NG108-15 a hybrid cell line (NG, 50 cells/spheroid). The cell ratios indicates that each spheroids contains ten times more quantity of SCs cells than NG cells, this could be attributed to enhance the spheroid environment with GFs secreted by SCs cells. The reason for the choice of cells, is that SCs and NG108-15 cells (which are neuroblastoma cross glioma cell line) complement well based upon the abilities of SCs to provide physical guidance axons and NG108-15 cells' ability to differentiate toward mature motor neuron phonotype.[177] The technique to developed the spheroids was hanging drops. The process starts with cell being centrifuged on non-adhesive wells, containing cell culture medium and 20% of methocel stock solution which prevents adhesion of cells and acts as an inert viscosity modulation substance. Methocel concentration is a parameter used to control de average size of the spheroids.[177] A clear collagen gel was develop and serve as the matrix in which the pellets containing the spheroids were introduce, finally obtaining the 3D co-culture as hanging drops on the collagen matrix, as shown in Fig. 12.

Figure 12. 3D co-culture, model of experimental setup. Spheroidal formation hanging drops and transfer in 3D collagen matrix. "Reprinted

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Neurite length was the response variable showed that in 2D culture is around 65 μm and the 3D model ranged from 115.56 to 176.6 μm, for 2.3× longer neurites.[69] The main reason for this behavior is that the model was able to increase the interactions between both cell types. To minimized cultivation differences between 2D and 3D cell culture both had a collagen layer. It is clear that no neurite growth into the collagen layer was noticed on 2D cell culture, which confirms that 3D setup was capable to increase cell–cell contacts and promote neurite growth.

A co-culture of SCs and ADSCs seeded on silk fibrin/collagen scaffold is an approach to develop a tissue-engineered nerve conduit (TENC).[20] The explanation for the choice of cells is that ADSCs are adult SCs that can be induced to neuropheres and neuronal-like cells when exposed to neurothrophic media, and this specific media is created by the presence of SCs. SCs have the capacity to secrete a variety of NTs, NGF, BDNF,GDNF, and others that help differentiate stem cells, making an ideal complement to promote and accelerated axonal growth. The 3D structure was develop by blending a solution of silk fibroin and collagen, at a ratio of 4 : 2, followed by cast molding and finished by lyophilization. It is important to mention that the conduit structure, stability, and biocompatibility, are dependent upon the choice of materials, for this case silk fibroin is added because of its excellent mechanical properties, which complements well with the biomcompatible properties of collagen.

The silk fibroin/collagen struture is seeded with the co-culture obtaning a TENC. Tissue regeneration was achieved on 1 cm long sciatic nerve defect in rats, which had results similar to autologous nerve grafts, but superior when compared with plain (non-co-culture scaffold). The conduit seeded with the co-culture mimics the native microenviroment, not only promoting the nerve regeneration, but also accelarating axonal growth as confirmed by the transdifferentiation of ADSCs when co-cultured with SCs. 3D models such as spheroidal co-culture and seeded co-culture on three dimensional scaffolds, projects to fill the gap between the monoculture assays and in vivo studies in order to represent cell–cell and cell–ECM complex interactions, offering a more detailed representation of physiological structures and conditions.[37, 95]

DRG cells are a mixed of SCs and neurons, and are categorized as a co-culture, and many experiments have been made using these cells. An example, is the use of a single walled carbon nanotube-composite hydrogel with electric stimulation, in which the neurite extension of the encapsulated DRG cells was evaluated. The hydrogel solution was composed of collagen type I and GF-reduced Matrigel®, in order to support SCs spreading, viability, and migration. It was proved that neurite extension was induced with the presence of the hydrogel and also by the electric stimulation; however, it is known that cells within the DRG, such as glia cells, may also influence in the results.[178] Another approach using DRG cells is with the combination of technologies of aligned PLLA microfibers with NGF releasing composite nanoparticles, both technique induced neurite extension with the presence of GF on nanoparticles and aligned microfibers.[164] Overall, DRG cells are a co-culture very representative of the response and performance necessary to reflect neurite extension.[164, 178] Hydrogels and scaffolds are platforms that offer 3D structures and co-culture simulate nerve cells models. Both aplications filled the

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gap between in vitro and in vivo experiments by being capable to mimic the native microenviemroment.

Validation of peripheral nerve three-dimensional cell culture models

Engineered polymeric scaffolds serve as highly tunable materials for 3D in vitro models of peripheral nerve. The 3D environment can more accurately recapitulate the native environment compared with 2D substrates, providing fundamental knowledge of neural tissue repair. 3D cell culture models are first validated by measuring adhesion of both neuron and glial cells, and by measuring neurite extension and glial cell proliferation. Additionally, protein and gene expression must be considered and can be evaluated on the 3D scaffolds. For example, Tuj1 is typically evaluated as an early marker for neuron development.[41, 54, 140, 165, 179] GAP43 and synapsin I, are both known to be upregulated throughout neuronal development.[180] For glia, during the transition of immature SCs toward myelinating, PMP22 and EGR2 are upregulated, while a down-regulation of NCAM and low affinity NGF receptor (LNGFR) occurs.[181] Also, adhesion of SCs to scaffolds may be validated by an overexpression of S100.[14, 19–21, 51, 53, 54, 56, 63, 66, 77]

However, evaluating a handful of expressed proteins and/or genes gives a limited picture of how the proposed 3D models are behaving in comparison with native tissue. A complete transcriptomic and/or proteomics profile of the in vitro 3D cell culture is crucial for the development of proper models that closely resemble native tissues. For instance, transcriptomic analysis allowed for the confirmation that engineered GF-induced neural progenitor cells closely resembled brain-derived neural progenitor cells.[182] Other work has used microarray analysis to identify new genes involved during peripheral nerve regeneration.[183] Recently, a proteomics analysis was performed to evaluate MSCs cultured in three similar ECM mimetic materials from different sources.[184] They observed that each material induced a unique matrisome signature, but they all shared a common set of proteins. Very little work has been published on validating 3D cell culture models of peripheral nerve using an omics approach. Carefully designed 3D environments must be validated via transcriptomic or proteomics analysis by comparing them to native cells or tissues to provide high fidelity to the 3D cell culture model. Lastly, high-throughput biomaterial screening platforms have emerged as useful strategies for the design and testing of biomaterials to serve as 3D environments for cell culture.[185]

Conclusions

The baseline for 3D cell culture platforms continues to expand. Many options are currently available to researchers. It is important to continue efforts and integrated strategies that not only can be executed at research laboratories but expedite the introduction of more effective therapies and sustainable treatments. There is still work to be done in the nerve regeneration field, especially to gain knowledge about improvements to overhaul many of the practical limitations, and what are the explicit points that remain departing from the nerve biology. 3D cell culture models may provide answers to these fundamental questions in neural tissue regeneration.

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Abbreviations	
1,6-bis (p-carboxy phenoxy) hexane	CPH
1,8 bis (p carboxyphenoxy) 3,6 dioxaoctane	CPTEG
Adipose-derived stem cells	ADSC
Additive manufacturing	AM
Bone marrow mesenchymal stem cells	BMSC
Brain-derive neurotrophic factor	BDNF
Cell adhesion molecules	Cams
Central nervous system	CNS
Chitosan and poly lactic-based co-polymers	CS-PLLA
Chitosan nerve guides	CNGs
Cryopolymerized gelatin methacryloyl	cryoGelMA
Dibasic sodium phosphate	DSP
Digital micro-mirror arrays devices	DMD
Dorsal root ganglia	DRGs
Early growth response 2	EGR 2
Extracellular matrix	ECM
Fast Fourier transform	FFT
Food and Drug Administration	FDA
Fused deposition modeling	FDM
Gelatin methacryloyl	GelMA
Genipin	A/GL_GP
Glial cell-derived neurotrophic factor	GDNF
Glycodoxypropyltrimethoxysilane	GPTMS
Glycosaminoglycans	GAGs
Gold nanoparticles	GNP
Growth factors	GFs
Growth-associated protein	GAP43
Hyaluronan tetrasaccharide	Ha4
Hyaluronic acid/hyaluronan	HA
Induced pluripotent stem cells	iPSC
Laminin	LN
low affinity NGF receptor	LNGFR
Mesenchymal stem cells	MSCs
Mouse embryonic stem cells	mESCs
Multicellular spheroids	MCS
Neonatal olfactory bulb ensheating Cells	NOBECs
Nerve growth factor	NGF
Nerve guide conduits/nerve guide channels	NGCs
Neuronal cells/ar adhesion molecules	NCAM
Neurotrophin	NT
Neurotrophin 4/5	NT-4/5
Neurotrophin-3	NT-3
Olfactory ensheathing cells	OECs

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Olfactory ensheathing cells	OECs
Peripheral nervous system	PNS
Poly(chitosan-g-lactic acid)	PCLA
Poly-ε-caprolactone	PCL
Poly-ethylene glycol	PEG
Poly (ethylene glycol) diacrylate	PEGDA
Polyethylene oxide	PEO
Poly-lactic-co-glycolic acid	PLGA
Poly-L-lactic acid	PLLA
Polypyrrole	Ppy
Polyvinyl alcohol	PVA
Rapid prototyping	RP
Scanning electron microscopy	SEM
Schwann cells	SC
Schwann cells peripheral myelin protein 22	PMP 22
Schwann-like cells	SCLs
Human neuroblastoma cells	SH-EP and/or SH-SY5Y Cells
Structured light scanning	SLS
Primary superior cervical ganglion neurons	SCG
Tissue-engineered nerve conduit	TENC

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