Co-culture methods to study neuronal function and disease

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Co-culture methods to study neuronal function and disease: Injury, infection and degenerative diseases can occur throughout the body, potentially causing impaired organ function, loss of sensation, increased pain and/or tissue death. Recently, researchers have begun to investigate ways to better mimic organ physiology by culturing neurons with the cell-type(s)-of-interest toward the goals of regenerating organs that accurately communicate with autonomic and sensory-somatic nervous systems. Co-culture research makes it possible to study the attraction, migration, proliferation, and differentiation resulting from the crosstalk between different cell populations. Co-culturing cells can sometimes facilitate more in-depth studies about an innervated organ than would be possible with *in vivo* research. For instance, tissue complexity and technical difficulties challenged research into bladder innervation until a co-culture assay with dorsal root ganglion neurons and urothelial cells provided an *in vitro* solution (O'Mullane et al., 2013). Understanding cell crosstalk will be particularly important to repair nervous system injuries or treat nervous system diseases. Some clinicians are already testing mesenchymal stem cell treatments to repair spinal cord injuries or treat Alzheimer's disease. While the initial findings have been promising, a better understanding of the molecular mechanisms underlying these processes will help optimize treatment. Here, *in vitro* co-culture assays using neuronal and non-neuronal cell populations are highlighted to provide a "toolbox" for researchers to determine how to best address their research questions and objectives. The special requirements of each system and the various readouts for each assay are discussed, with focus placed on what parameters can be investigated with each method.

Media, growth factors, substrate mechanics and dimensions all affect cellular responses in monoculture as well as in co-culture. Co-cultured cells additionally respond to co-habitant cells, including intercellular and extracellular dynamics and paracrine signaling. Recent research demonstrated greater neurite outgrowth when neurons were co-cultured with dental pulp stem cells (DPSCs) than with bone marrow stem cells (BMSCs) (Pagella et al., 2020), indicating that neurites can differentiate between seemingly similar cells and respond accordingly. Furthermore, proliferation and differentiation occur on different timelines depending on the cell type and *in vitro* conditions. This can prevent neurite outgrowth in some co-cultures if the proper reagents are not provided (Barkley et al., 2020) or may require long culture periods to attain the desired differentiation of the tissue (Clark et al., 2017). The cell culture parameters and special requirements of the co-culture assays should be taken into consideration when designing experiments. This perspective provides an overview of culture strategies, organized from simple to increasingly sophisticated techniques, with **Additional Table 1** outlining the main strengths and weaknesses of each technique. These assays can be used to study organ development and repair, perform drug screens, and investigate disease pathologies that better recapitulate *in vivo* conditions than monocultures. They are therefore more predictive models of *in vivo* responses.

One plate for two populations in direct contact: Primary stem or immortalized cells and neurons or ganglia can be plated together in a simple co-culture assay (**Figure 1A**). This method has been used to study axoglial communications by co-culturing Schwann cells atop human induced pluripotent stem cell-derived neurons, and was applied to demonstrate how certain pharmacological agents led to demyelination and axonal degeneration (Clark et al., 2017). These simple co-cultures can be used to screen drugs and study several nervous system diseases. Unfortunately, these assays become more complicated when less similar cells are co-cultured. This is because media for one cell population may not support the other population, or one population may require growth factors at different concentrations than are needed for the other population *in vivo*. In these situations, media optimization is necessary, which requires identifying which components are crucial to each cell type and which mixture (and concentrations) best sustains both populations. The initial cell growth could also be performed in independent chambers with the respective media before the barrier is removed to allow the media and cell populations to combine. If this still does not provide adequate nutrition, a microfluidic platform, as described below, can maintain each cell population in an independent chamber with its optimized media. It is important to note that neuronal responses in such direct contact co-cultures result from a complicated mixture of paracrine and intercellular signaling that cannot be differentiated with this method. However, hydrogels can begin to separate these contributions.

Hydrogels to improve co-culture conditions: Hydrogels offer the opportunity to suppress signals from the co-culture media in a direct-contact model to improve studies of intercellular signaling. This method, depicted in **Figure 1B**, has been used for neurons coated with an agarose gel that allowed direct contact with gustatory cells cultured atop the gel. The gel dampened the neuronal responses to calcium fluxes from the media, and allowed for more precise quantification of the calcium ion responses due to intercellular signaling (Le-Kim et al., 2019). The hydrogel architecture can also provide a favorable three-dimensional (3D) matrix for tissue development. In one report, a microporous hydrogel facilitated the development of innervated vascular networks capable of microcirculation when implanted into an animal model (Ford et al., 2006). Given the progress in hydrogel techniques, it is now possible to engineer materials containing specific ligands, at an optimal density, as well as to fine-tune the gel porosity and elasticity to recreate an *in vivo*-like environment for the co-cultured cells. Before designing experiments, the crosslinking methods and material components must be carefully assessed to avoid possible toxic effects. It should also be kept in mind that while these modulations can create more *in vivo*-like conditions, the origin of the extracellular matrix is the cells themselves. Engineered matrices only control the initial conditions, which are then modified by the adhered or encapsulated cells. While this complicates the data analysis, it also creates an opportunity to perform initial assessments of new materials that could eventually be injected or implanted with encased cells or tissues to treat injuries or various diseases.

Transwell filter atop a tissue culture plate: Paracrine signals guiding neurite outgrowth can be studied using the Transwell co-culture setup (**Figure 1C**). Non-neuronal cells can be cultured in a tissue culture plate well while neurons are cultured atop an overlying Transwell filter (or *vice versa*). Neurite outgrowth toward the non-neuronal cells is facilitated by 3 mm filter pores. The distance between cell populations prevents intercellular and synaptic signaling. Thus, the neurite outgrowth is directly related to paracrine signaling. Although this method does not require the addition of neuronal growth factors to the media, mitotic inhibitors may be necessary to prevent overgrowth. The impact of these treatments should be considered when designing experiments using this system. Multiple measurements can be performed using this method, including: 1) cell RNA/protein quantification using PCR/western blot on cell lysates, 2) quantification and characterization of neurite outgrowth using immunofluorescence or crystal violet staining, 3) evaluation of cell morphology and identity changes in response to TG or dorsal root ganglion neuronal secretions using immunofluorescence on cells plated below neurons, and 4) paracrine signal identification and quantification using enzyme-linked immunosorbent assay or proteomic analyses on collected media. Cell signaling in either population can be inhibited using a variety of methods, and ectopic growth factors can be added to further elucidate the signaling pathways guiding neurite outgrowth. For instance, TG neurons co-cultured with DPSCs demonstrated increased neurite outgrowth compared to when the cells were grown alone or with other mesenchymal cells. However, if transforming growth factor beta signaling was attenuated in the DPSCs by the addition of a transforming growth factor beta inhibitor, this increase was not observed (Barkley et al., 2020). In another instance, astrocytes adhered to the overlying filter with neurons below were co-cultured to test the specific cytotoxicity of neurotoxic compounds (De Simone et al., 2017). This assay can typically be maintained for up to 5 days, but can prove expensive due to the cost of the growth factors and inhibitors if longer assays are desired.

Microfluidic chambers for co-culture: Because culture media are optimized for each cell type, the use of shared media in the aforementioned setups may create suboptimal conditions for either or both populations in the co-culture. A microfluidic platform contains separated chambers connected by microgrooves, in which the hydrostatic pressure created by volume differentials sustains media separation but still permits neurite outgrowth (and cell interactions) via the microgrooves. This platform allows the culture of each cell type in its own culture media. Ganglia or dispersed neurons must be cultured first to allow neurites to extend into and through the microgrooves (**Figure 1D**). One group of researchers used this platform to compare neurite outgrowth patterns induced by different mesenchymal cells. Trigeminal or dorsal root ganglia were co-cultured with DPSCs or BMSCs to determine the differences in neuronal-mesenchymal responses. Both the trigeminal and dorsal root ganglia demonstrated more neurite outgrowth and more extensive axonal networks when co-cultured with the DPSCs in comparison to co-culture with BMSCs. This indicated that paracrine and intercellular signals from DPSCs provide superior stimulatory effects, suggesting that they might represent a better therapeutic opportunity for neuroregeneration (Pagella et al., 2020). Because the intercellular and paracrine signaling occurs in a chamber separate from the neuronal bodies, the microfluidic platform is the only device in which communications can be spatially distinguished. For instance, glial-derived neurotrophic factor applied locally to axons promoted local neurite outgrowth and neuromuscular junction formation, whereas retrograde transport of glial-derived neurotrophic factor activated pro-survival signals (Zahavi et al., 2015). The effects of neuronal activity have also been investigated with a microfluidic platform in which axons in a central compartment were allowed to grow into two separate lateral

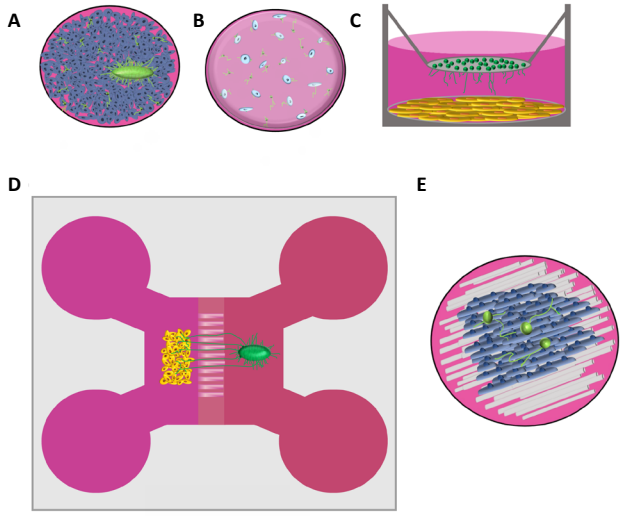

Figure 1 | **Co-culture systems used to study neurite outgrowth.**
(A) Neurons and ganglia (in green) are in direct contact and adhere to underlying non-neuronal cells (yellow) in one tissue culture plate. (B) Neurons encased in a hydrogel co-cultured with overlying non-neuronal cells. The hydrogel porosity slows media diffusion to highlight intercellular signaling. (C) Transwell filters with large pores allow neurite outgrowth from dispersed neurons to grow toward underlying non-neuronal cells in response to paracrine signaling. (D) A microfluidic platform prevents media mixing in the main chambers and allows neurite outgrowth through microgrooves, allowing them to interact with non-neuronal cells in a separate chamber. (E) Aligned nanofibers (in white) promote myotube (blue) formation to support the long-term culture of neurons that develop neuromuscular junctions with the underlying myotubules. The Figure is only intended to illustrate representative systems, and is not drawn to scale. A vast array of other set-ups can be made based on these systems to test specific experimental questions.

compartments, with neuronal activity inhibited by a gamma-aminobutyric acid type A receptor agonist in one compartment. The central component extended longer axons and formed more synapses with the uninhibited neurons, demonstrating that the neuronal activity in one population can affect a separate population (Coquinco et al., 2014). This finding might have clinical relevance for patients suffering from central nervous system diseases or disorders.

By combining several co-culture methods, researchers recently created a 3D triculture model to examine multiple variables. A fibrin-Matrigel mixture was injected and polymerized in the central channel between microfluidic chambers to provide a 3D extracellular matrix. Brain microvascular endothelial cells and BMSCs were co-cultured in one channel of a microfluidic platform and neural stem cells were cultured in the other channel to separate neurogenesis and angiogenesis. Researchers were able to test multiple gel and cell combinations to construct a 3D neurovascular tissue with which to study brain functions, screen drugs, and develop therapeutic strategies (Uwamori et al., 2017). The co-culture was performed for 3 days with daily media changes, which allowed for investigations into neurite outgrowth up to stage 3. A larger platform would be necessary to extend culture periods and allow the cells to condition the media. However, researchers could capitalize on the low media volume requirements to develop cost-effective and/or large-scale drug or growth factor screens targeting either or both cell populations. Alternatively, it could be used to determine the spatiotemporal requirements of chemotherapeutics that promote tissue repair or recovery in injured or diseased organs.

Nanofiber setups for co-culture: Every tissue has a highly specific architecture, which researchers strive to provide in cell culture studies. The 3D hydrogel microchamber provides a remarkable environment to construct and study neurovascular tissue, but more complex organs require more elaborate techniques. Electrospun nanofibers have been widely utilized in tissue engineering to design artificial extracellular matrices. In one set of experiments, random and aligned polylactic acid scaffolds were utilized to encourage C2C12 myoblasts to attach and form myotubes. Primary embryonic motor neurons from Sprague-Dawley rats were seeded atop the myotubes (**Figure 1E**). Significantly more myotubes formed on aligned versus randomly-spun scaffolds, which facilitated better axon alignment and elongation. Both scaffolds supported the formation and maintenance of neuromuscular junctions for up to 49 days, whereas cell viability ended at 14 days on glass substrates (Luo et al., 2018). This demonstrated that matrix cues enhance intercellular communication in co-cultures. Scaffolds have also been used to study the growth and survival of neuroblastoma and glioblastoma cell lines in co-culture to evaluate the effects of Parkinson's disease mimetics. The increased neuronal survival demonstrated with co- *versus* mono-culture (Chemmarappally et al., 2020) suggested that nanofiber scaffolds may be utilized to simulate cell-specific environments and study tissue damage and regeneration models that could be used for future drug screens.

Summary: Several co-culture techniques have been developed to answer research questions, with both the cultures and questions ranging from simple to complex in nature. Intercellular communication, paracrine signaling, synaptic signaling, chemical and physical cues from the extracellular environment, signal attenuation or enhancement and spatial distinction can all be assessed depending on the assay chosen. With recent improvements in biomaterials, genetic techniques and fundamental knowledge of organ dynamics, the development of a functional (innervated), regenerated organ is due to progress at a rapid pace. Future research will capitalize on combining multiple techniques, such as patterning a microfluidic chamber to promote accurate tissue construction similar to that described above with scaffolds. Therapeutic strategies can be fine-tuned by the ability to assess time- and concentration-related gradients of chemotherapeutics. While each of these techniques have unique attributes that make them useful for specific studies, the low volume requirements and capacity to integrate automation into the microfluidic platform could soon be utilized in large-scale studies to expedite the progress and reduce the costs of bench-to-bedside research.

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Additional files:
Additional file 1: *Open peer review reports 1 and 2.*
Additional Table 1: *Main strengths and weaknesses of different culture techniques.*

References

Barkley C, Serra R, Peters SB (2020) A co-culture method to study neurite outgrowth in response to dental pulp paracrine signals. J Vis Exp doi:10.3791/60809.
Chemmarappally JM, Pegram HCN, Abeywickrama N, Fornari E, Hargreaves AJ, De Girolamo LA, Stevens B (2020) A co-culture nanofibre scaffold model of neural cell degeneration in relevance to Parkinson's disease. Sci Rep 10:2767.
Clark AJ, Kaller MS, Galino J, Willison HJ, Rinaldi S, Bennett DLH (2017) Co-cultures with stem cell-derived human sensory neurons reveal regulators of peripheral myelination. Brain 140:898-913.
Coquinco A, Kojic L, Wen W, Wang YT, Jeon NL, Milnerwood AJ, Cynader M (2014) A microfluidic based in vitro model of synaptic competition. Mol Cell Neurosci 60:43-52.
De Simone U, Caloni F, Gribaldo L, Coccini T (2017) Human co-culture model of neurons and astrocytes to test acute cytotoxicity of neurotoxic compounds. Int J Toxicol 36:463-477.
Ford MC, Bertram JP, Hynes SR, Michaud M, Li Q, Young M, Segal SS, Madri JA, Lavik EB (2006) A macroporous hydrogel for the coculture of neural progenitor and endothelial cells to form functional vascular networks in vivo. Proc Natl Acad Sci U S A 103:2512-2517.
Le-Kim TH, Koo BI, Yun JS, Cho SW, Nam YS (2019) Hydrogel skin-covered neurons self-assembled with gustatory cells for selective taste stimulation. ACS Omega 4:12393-12401.
Luo B, Tian L, Chen N, Ramakrishna S, Thakor N, Yang IH (2018) Electrospun nanofibers facilitate better alignment, differentiation, and long-term culture in an: In vitro model of the neuromuscular junction (NMJ). Biomater Sci 6:3262-3272.
O'Mullane LM, Keast JR, Osborne PB (2013) Co-cultures provide a new tool to probe communication between adult sensory neurons and urothelium. J Urol 190:737-745.
Pagella P, Miran S, Neto E, Martin I, Lamghari M, Mitsiadis TA (2020) Human dental pulp stem cells exhibit enhanced properties in comparison to human bone marrow stem cells on neurites outgrowth. FASEB J 34:5499-5511.
Uwamori H, Higuchi T, Arai K, Sudo R (2017) Integration of neurogenesis and angiogenesis models for constructing a neurovascular tissue. Sci Rep 7:17349.
Zahavi EE, Ionescu A, Gluska S, Gradus T, Ben-Yaakov K, Perlson E (2015) A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. J Cell Sci 128:1241-1252.

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Additional Table 1 Main strengths and weaknesses of different culture techniques

Assay	Main Strengths	Main Weaknesses
A	Simple, inexpensive method to begin a study and establish a foundation of knowledge.	Cell media is shared and therefore cannot be optimized for each individual population.
	Assay can be extended for several weeks, with multiple media changes.	Intercellular and paracrine signal effects cannot be independently studied.
B	Paracrine signaling can be dampened to allow a more direct investigation of intercellular signaling.	Crosslinking reagents or methods can be cytotoxic.
	Hydrogel mechanics and ligand identity and density can be optimized to provide a more in vivo-like environment.	Artificial matrices only control the initial conditions and cannot be dynamically modified by the researcher.
	Hydrogels containing cells or tissues can be injected or implanted *in vivo* to treat a disease or repair an injury.	Cell media is shared and therefore cannot be optimized for each individual population.
	Hydrogels can be incorporated into other techniques to add dimensions.	Intercellular and paracrine signal effects cannot be completely isolated for independent study.
C	Both cell populations can be genetically modified independently.	Only paracrine signaling can be investigated.
	Cell lysates can be independently collected to study transcriptional and translational responses to experimental variables.	Cell media is shared and therefore cannot be optimized to each individual population.
	Media can be collected and analyzed to investigate paracrine signaling between cell populations.	Long-term studies could prove expensive due to the need for specific growth factors or inhibitors.
D	Separated chambers allow optimized media to be used for each cell population.	Ganglia/neurons need to be pre-cultured to allow axons to extend through microchannels before co-culturing begins.
	Microfluidic platforms can reduce media volumes, and thereby reduce experimental costs.	Techical replicates could prove expensive.
	Hydrogel matrices can be incorporated to design a three-dimensional triculture method.	
	The non-neuronal cell interactions with axons and neuronal bodies can be studied separately.	
	Synaptic signaling between multiple neuronal populations can be studied with multi-chamber platforms.	
E	Assays can be extended much longer than using other techniques.	Neurons may sense and respond to the mechanics of underlying artificial matrices.
	In vivo-like cellular/tissue architecture can be pre-established to investigate its effects on neurite outgrowth.	Cell media is shared, and therefore cannot be optimized for each cell population.
	Scaffolds or patterning can be incorporated into the microfluidic platform.	Intercellular and paracrine signal effects cannot be independently studied.