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Title Page:

The Emerging Role of Neuronal Organoid Models in Drug Discovery: Potential Applications and Hurdles to Implementation

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Running Title Page:

Running Title: Neuronal Organoid Models in Drug Discovery

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Nonstandard Abbreviations:

3D automated reporter quantification (3D-ARQ)

embryoid body (EB)

embryonic stem cells (ESCs)

extracellular matrices (ECMs)

fluorescent light sheet microscopy (FLSM)

hit identification (Hit ID)

immunohistochemistry (IHC)

induced pluripotent stem cells (iPSCs)

lead identification (LID)

lead optimization (LO)

multi-electrode arrays (MEAs)

research operating plans (ROPs)

structure activity relationships (SAR)

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Abstract:

The high failure rate of drugs in the clinical pipeline is likely, in part, the result of inadequate preclinical models, particularly those for neurological disorders and neurodegenerative disease. Such preclinical animal models often suffer from fundamental species differences and rarely recapitulate all facets of neurological conditions, while conventional two-dimensional (2D) *in vitro* models fail to capture the three-dimensional (3D) spatial organization and cell-to-cell interactions of brain tissue that are presumed critical to the function of the central nervous system. Recent studies have suggested that stem cell-derived neuronal organoids are more physiologically-relevant than 2D neuronal cultures, due to their cytoarchitecture, electrophysiological properties, human origin, and gene expression. Hence, there is interest in incorporating such physiologically-relevant models into compound screening and lead optimization efforts within drug discovery. However, despite their perceived relevance, compared to previously utilized preclinical models, little is known regarding their predictive value. In fact, some have been wary to broadly adopt organoid technology for drug discovery due to the low throughput and tedious generation protocols, inherent variability, and lack of compatible moderate-to-high throughput screening assays. Consequently, microfluidic platforms, specialized bioreactors, and automated assays have been, and are being, developed to address these deficits. This mini review provides an overview of the gaps to broader implementation of neuronal organoids in a drug discovery setting, as well as emerging technologies that may better enable their utilization.

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Significance Statement:

Neuronal organoid models offer the potential for a more physiological system in which to study neurological diseases, and efforts are being made to employ them, not only in mechanistic studies, but also in profiling/screening purposes within drug discovery. In addition to exploring the utility of neuronal organoid models within this context, ongoing efforts in the field aim to standardize such models for consistency and adaptation to standard screening platforms for more throughput evaluation. This mini review covers potential impact of and hurdles to implementation for these models.

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Mini Review:

Neurological conditions, including neurodegenerative diseases, neurodevelopmental disorders, and injury to the nervous system, remain a significant, unmet burden to the United States healthcare system. In 2014 alone, these conditions affected over 100 million Americans and cost nearly 800 billion dollars (Gooch *et al.,* 2017; Jensen *et al.*, 2018; Papariello and Newell-Litwa, 2020). Specifically, in 2017 it was estimated that Parkinson's Disease (PD) affected 1 million Americans and cost 52 billion dollars (Yang *et al.,* 2020). In 2020, Alzheimer's Disease (AD) affected 5.8 million Americans and, along with other dementias, was estimated to cost 305 billion dollars (2020 Alzheimer's disease facts and figures, 2020). Furthermore, as the size of the country's elderly population will nearly double by 2050, these numbers are predicted to increase substantially (Gooch *et al.,* 2017). Despite the large demand for improved neurological care, the greater than 90% failure rate of drugs in the clinical pipeline has resulted in a dearth of pharmaceutical treatments for these conditions (Jensen *et al.*, 2018; Hingorani *et al.*, 2019; Papariello and Newell-Litwa, 2020). These clinical failures often occur after billions of dollars and years of development have been invested in a potential new drug (Breslin and O'Driscoll, 2013; Hung *et al.,* 2017).

The poor translatability of promising preclinical compounds to the clinic is partially the consequence of inadequate preclinical models of neurological disease. Typical preclinical compound evaluation involves *in vitro* assessment of the target, followed by *in vivo* assessment of pharmacodynamic effect in at least one non-human species. In the case of neurological conditions, researchers have struggled to reliably recapitulate disease pathology within historical *in vitro* and *in vivo* models due, in part, to limited understanding of the underlying pathological mechanisms (Charvin *et al.*, 2018). Furthermore, conventional models of human origin in a

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monolayer are not representative of the physiological environment of the brain: they lack the relevant cytoarchitecture, spatial organization, and cell-to-cell interactions that are crucial to the function of the nervous system. Therefore, they have shown limited efficacy in the exploration of disease mechanisms and in the screening of neuroprotective agents (Schüle *et al.*, 2009; Baden *et al.*, 2019). As with *in vitro* models, *in vivo* models have also failed to appropriately recreate neurological disorders. Modest conservation of genome sequences across species, as well as fundamental differences in brain development and function, have resulted in animal models that often only reproduce a few facets of the human disease (Dawson *et al.*, 2018). Furthermore, the limited lifespan of many animals may prevent the development of pathological mechanisms that are associated with age-related neurodegenerative diseases, including PD and AD (Dawson *et al.*, 2018).

Recent studies suggest that three-dimensional (3D) tissue models derived from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) reproduce relevant complex physiology that may make them superior to both *in vivo* and conventional *in vitro* preclinical models for the interrogation of human neurological disorders (Zhang *et al.*, 2014; Jo *et al.*, 2016; Lee *et al.*, 2016; Centeno *et al.*, 2018; Liu *et al.*, 2018; Trujillo *et al.*, 2019). Such 3D models include organoids, spheroids, tissue-engineered constructs, bio-printed tissue, microfluidic systems, and organ-on-a-chip technologies. For the purposes of this review, we will focus on the potential utility of organoid technology in drug discovery; the use of other 3D tissue models has been reviewed elsewhere (Sanders *et al.*, 2014; Fang and Eglen, 2017; Haring *et al.*, 2017; Thomas and Willerth, 2017; Langhans, 2018; Antill-O'Brien *et al.*, 2019; Frimat and Luttge, 2019). We also want to discriminate between neuronal organoid and neuronal spheroid models, which are frequently confused (Figure 1A). While both are 3D models generated

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through the controlled differentiation of stem cells, organoids are larger (up to 4 mm in diameter), and they demonstrate self-organization of tissue into discrete cellular layers. Furthermore, as described below, organoid fabrication protocols frequently utilize externally applied extracellular matrices (ECMs) to support tissue outgrowth. Spheroids, on the other hand, are small (~500 μm in diameter), do not exhibit self-organization, and do not require the use of ECMs (Zhuang *et al.*, 2018).

Briefly, a general neuronal organoid fabrication protocol involves first aggregating stem cells into a cell mass called an "embryoid body" (EB). Next, a series of growth factors and/or small molecules are applied to differentiate the EB towards the appropriate lineage. Finally, the growing organoid is encapsulated within an ECM, which promotes further tissue outgrowth and maturation over months *in vitro* (Figure 1B). Through their own self-assembly, neuronal organoids develop complex three-dimensional structures, including proliferative zones that closely imitate the cytoarchitecture of the neural tube during development (Lancaster and Knoblich, 2014a; b; Wang, 2018). Such structures may encourage cell morphology, signaling, and electrophysiological properties within the organoids that are more representative of brain tissue than corresponding features in traditional 2D cultures (Breslin and O'Driscoll, 2013; Pasca *et al.*, 2015; Jo *et al.*, 2016; Tekin *et al.*, 2018; Wang, 2018; Ao *et al.*, 2020). Neuronal organoids are also capable of proactively producing and self-organizing distinct neuronal subtypes, as well as astrocytes and oligodendrocytes that are crucial for intrinsic neuronal function (Lancaster and Knoblich, 2014a; Dezonne *et al.*, 2017; Hyosung Kim *et al.*, 2019; Marton *et al.*, 2019). It should be noted, however, that current neuronal organoid models do not intrinsically develop microglia or vascular systems (Lee *et al.*, 2017). Furthermore, analyses demonstrate that neuronal organoids more closely resemble human brain tissue, compared to traditional 2D iPSC-derived

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neuronal cultures (Jo *et al.*, 2016). However, with that said, gene expression analyses show that neuronal organoids most closely align with prenatal brain tissue rather than adult, which is not ideal when using organoids to study age-related neurodegenerative diseases (Jo *et al.*, 2016). Thus, in order to "age" these models for greater applicability to such diseases, researchers are exploring the application of oxidative stress to accelerate pathogenic mechanisms associated with age (Grenier *et al.*, 2020).

Such attractive properties have prompted researchers to generate organoid models of many neurodevelopmental disorders and neurodegenerative diseases, including microcephaly, macrocephaly, autism, schizophrenia, AD, PD, and dementia (Lancaster *et al.*, 2013; Mariani *et al.*, 2015; Li *et al.*, 2017; Seo *et al.*, 2017; Ye *et al.*, 2017; Amin and Paşca, 2018; Park *et al.*, 2018; Wang, 2018; Hongwon Kim *et al.*, 2019). However, despite their improved physiological relevance, the utility of organoids to screen preclinical compounds and potentially better predict clinical efficacy remains unclear. Poor reproducibility, low throughput generation, and a lack of amenable 3D assays have limited their widespread adoption in the context of drug discovery. These hurdles must be fully recognized and addressed in order to ascertain their predictive value for compound profiling.

As with other complex 3D models, organoids suffer from both within-batch as well as between-batch variability in size and cellular composition (Quadrato *et al.*, 2017; Yakoub and Sadek, 2018). Yakoub et al. showed that organoid diameter within a batch can differ by several millimeters, while Quadrato et al. used single-cell RNA sequencing to determine that organoids from separate batches produced varying neuronal subtypes (Quadrato *et al.*, 2017; Yakoub and Sadek, 2018). As neuronal organoid self-assembly is highly dependent upon the precise application of small molecules and growth factors in appropriate intervals, minute variations in

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any of these factors can potentially affect stem cell differentiation and lead to downstream inconsistencies. In addition to growth media, extracellular matrix composition, environmental factors (i.e. temperature and humidity), and the initial number of cells seeded can influence both organoid size and maturation (Booij *et al.*, 2019). This variability presents a major hurdle to the implementation of organoids for both traditional and phenotypic screening applications, as well as target validation efforts. For example, compounds may penetrate organoids of different sizes at different rates and to different depths, potentially leading to confounding assay outcomes, depending on the readout. As drug discovery research operating plans (ROPs) are built upon the premise that *in vitro* assay readouts have sufficient relative predictive resolution regarding a compound-mediated effect to guide structure activity relationships (SAR), organoid generation must be standardized prior to their use as screening tools to reduce the likelihood of unreliable results.

Automation, fate-restricting differentiation protocols, 3D bioprinting, and tissue engineering technologies could potentially assist with improving organoid homogeneity (Table 1). As the size of the initial EB cell mass plays a crucial role in organoid development, many researchers are no longer relying upon spontaneous cellular aggregation to form their EBs. Rather, the centrifugation of stem cells into patterned micro-wells and V-shaped well plates is being utilized to improve EB consistency between batches (Ungrin et al., 2008). In particular, Ungrin et al. found that the centrifugation method reduced their coefficient of variation for EB size from 0.72 to ~0.09. Similarly, several groups have built microfluidic platforms that confine growth volume in order to generate organoids of more uniform size (Zhu *et al.*, 2017; Ao *et al.*, 2020). Ao et. al achieved a coefficient of variation of 0.087 for mature organoid size by restricting their perfusable growth chamber to 2 mm in diameter (Ao *et al.*, 2020).

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In order to reduce inconsistencies in cellular composition between neuronal organoids, numerous (>15) differentiation protocols have been generated that restrict cell lineage fate. For example, Qian et al. demonstrated that pre-patterning their EBs with fate-restricting dual SMAD inhibitors decreased tissue heterogeneity (Qian *et al.*, 2016). The dual SMAD inhibition functioned to prevent differentiation of EBs towards a non-neural fate and to push them towards a neuroectodermal lineage (Qian *et al.*, 2016). Retinoic acid has also commonly been used to promote differentiation towards a neuronal lineage (Schuldiner *et al.*, 2001). Along these same lines, Monzel et al. used fate-restricted neuroepithelial stem cells, rather than pluripotent stem cells, to initiate organoid production for more efficient differentiation toward the midbrain (Monzel *et al.*, 2017).

Three-dimensional bioprinting has been proposed as a method to regulate organoid cytoarchitecture and self-assembly via precise placement of extracellular matrix hydrogels and/or individual cells (Silva *et al.*, 2019). For example, hydrogel scaffolds that impose complex 3D structures for tissue engineering applications have been produced by nozzle, laser, and inkjet printing systems (Cui *et al.*, 2012; Hinton *et al.*, 2015; Jang *et al.*, 2018; Silva *et al.*, 2019). In addition to printing biomaterials, 3D printers have the capacity to deposit individual cells. Although not in the context of a neuronal model, Fedorovich et al. used 3D fiber deposition to generate osteochondral tissue by printing chondrocytes and osteogenic progenitors in separate compartments of an alginate scaffold. The separate compartments developed into distinct tissue types and expressed different protein markers (Fedorovich *et al.*, 2012). These existing 3D printing capabilities could be adapted to guide consistent cytoarchitecture organization within embryoid bodies.

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In addition to automation, fate-restriction, and 3D bioprinting, the use of spatiotemporally defined differentiation cues has also been proposed as a means to control neuronal organoid fate more closely (Silva *et al.*, 2019). Morphogen gradients generated by microfluidic injection, slow-releasing micro-beads, and bioactive scaffolds have all been considered (Mahoney and Saltzman, 2001; Uzel *et al.*, 2016; Lancaster *et al.*, 2017; Sun *et al.*, 2018; Karzbrun and Reiner, 2019; Silva *et al.*, 2019). Uzel et al. previously demonstrated they could establish linear biochemical concentration gradients within their microfluidic chambers in order to closely control the neuronal differentiation of 3D tissue constructs. Their device relies upon diffusion of molecules across inlet and outlet ports to generate gradients, which are naturally occurring during development and direct differentiation of developing tissues (Uzel *et al.*, 2016). Likewise, Mahoney et al. incorporated microparticles within cell aggregates transplanted into the brain for the purpose of establishing a "synthetic micro-environment." The microparticles released nerve growth factor over time to generate a local concentration gradient and promote targeted differentiation (Mahoney and Saltzman, 2001). Finally, Wylie et al. used light patterning to immobilize growth factors at certain locations within a 3D hydrogel in order to direct stem cell differentiation. This technique utilizes two-photon irradiation to expose reactive chemical groups within the hydrogel that are then able to bind and immobilize growth factors (Wylie *et al.*, 2011). In addition to morphogen gradients, mechanical cues may be utilized to regulate stem cell differentiation within neuronal organoids (Silva *et al.*, 2019). For example, Przybyla et al. showed that matrix stiffness influenced activation of differentiation pathways in hESCs, with more compliant matrices enhancing mesodermal differentiation (Przybyla *et al.*, 2016). Many ECM attributes can be adjusted and/or incorporated to control mechanical cues in a spatiotemporal manner, including crosslinking density, photodegradable hydrogels, and

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enzymatic cleavage points (Raeber *et al.*, 2005; Kloxin *et al.*, 2009; Silva *et al.*, 2019). Yoshikawa et al. generated a pH-sensitive hydrogel that could be exploited to tune the elasticity of cell-laden scaffolds (Yoshikawa *et al.*, 2011). Therefore, spatiotemporal control of differentiation cues could also be employed to direct uniform differentiation of stem cells within organoids.

Finally, in order to eliminate inconsistencies in environmental conditions across preparations, the use of regulated bioreactors to closely control temperature, humidity, and mechanical forces has been suggested (Quadrato *et al.*, 2017). Due to the significant lot-to-lot variations in composition and concentration observed for naturally-derived growth factors, serum, and extracellular matrices, it also may be advisable to buy such components in bulk. Such a practice could reduce inconsistencies in organoid growth media across batches. Alternatively, differentiation protocols that eliminate the need for growth factors are also being developed. For example, Reinhardt et al. established a protocol to generate neural precursor cells from stem cells using only small molecules (Reinhardt *et al.*, 2013). Likewise, synthetic hydrogels could potentially replace biological hydrogels within the organoid fabrication process to avoid between-batch variability (Langhans, 2018).

Due to the many sources of variability within neuronal organoids, the field could benefit from the creation of a committee capable of providing guidelines and best practices for both the generation and use of neuronal organoids. At a recent panel discussion on “Organoid Quality Control” at an EMBL Symposium focused on organoid technology, academic and industry experts stressed the need for standardization and transparency of protocols across the burgeoning organoid field (EMBO | EMBL Symposium: Organoids: Modelling Organ Development and Disease in 3D Culture, 2020). For example, many scientists incorrectly use the terms “spheroid”

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and “organoid” interchangeably, and default metrics for organoid quality control are unclear. Additionally, there is confusion over what constitutes a biological versus technical organoid replicate, and whether isogenic controls are necessary. A guidance committee could help normalize organoid work across institutions by creating defined criteria for nomenclature, publishing optimized differentiation protocols, and even potentially providing organoid standards for external comparison. Such normalization across the field would help enhance the quality of research by allowing for increased transparency and improved communication.

Along with reproducibility, throughput generation remains a major hurdle to the implementation of organoids in a drug discovery setting. There are several phases within early drug discovery efforts where organoids could be utilized, which would require higher throughput organoid production for sufficiently powered experiments (Figure 2). Typically, a preclinical drug discovery ROP is used to triage and refine targeted compounds of interest and encompasses hit identification (Hit ID), lead identification (LID), and lead optimization (LO) efforts. Hit ID helps narrow the population of active chemistries. During this process, moderate-to-high throughput screening campaigns ultimately deliver a package containing active compounds (hits) derived from a library. Such campaigns may involve single point hit identification, followed by multiple-point titration confirmation efforts, among other evaluations. As even relatively small compound libraries are typically comprised of thousands to tens of thousands of compounds, one can begin to see how the numbers of organoids required to support even a small screening campaign would add up quickly. Therefore, while theoretically applicable for screening campaigns, organoid implementation in LID efforts would be far more practical, currently. This phase of the ROP explores SAR tractability within various chemical classes, allowing for determination of the most promising lead series. Here specifically, organoids could replace, or

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serve as orthogonal assays to, 2D cell-based assays, assessing on-target activity in a theoretically more physiologically-relevant system. Adequate organoid numbers would be required to support the compound flow at this stage in the ROP- likely at least hundreds, if not thousands of compounds, over the course of a program. Alternatively, if their value as a complex human model for predicting preclinical to clinical translation can be established, organoids could be placed toward the bottom of an ROP, to facilitate LO efforts. This phase of the ROP allows for establishment of an optimized preclinical candidate, where organoid models may add value orthogonal to traditional *in vivo* preclinical evaluation. Unfortunately, the majority of current protocols to generate organoids are low-throughput and expensive, requiring time-intensive manual intervention. Furthermore, as screens have historically been performed with 2D monolayer or suspension cultures, the infrastructure to operate with organoids may be lacking. For example, it is more difficult to program standard liquid handlers to manage 3D cultures, and such instrumentation is generally not ideal for working with viscous solutions such as the ECM hydrogels utilized to encapsulate EBs (Booij *et al.*, 2019).

Specialized bioreactors and, again, microfluidic platforms could be the ideal tools to increase organoid production capacity and reduce costs (Table 2). Several researchers are already utilizing microfluidic platforms to streamline organoid production and obviate the need for manual intervention. Wang et al. adapted technology previously devised for organ-on-a-chip platforms to continuously perfuse growing organoids with media, thus cutting the time required for media changes (Wang *et al.*, 2018). Ao et al. used microfluidic technology to seed stem cells, form embryoid bodies, induce differentiation, and encapsulate developing neuronal organoids in extracellular matrix (Ao *et al.*, 2020). This platform required minimal manual manipulation of organoids and no organoid transfers. It was also capable of producing 169 neuronal organoids in

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one 6-well plate, which is relatively high density compared to traditional manual protocols (Ao *et al.,* 2020). Specialized bioreactors are also being developed to minimize the volume of expensive growth media required to support organoid production. Qian et al. developed the "Spin Omega" bioreactor to provide suspension culture for organoids as an alternative to large spinning flasks. These bioreactors feature a small footprint, allow multiple test conditions to be investigated at once, and require minimal media. In fact, compared to the previously used spinning flasks that require 100 milliliters of media, each Spin Omega bioreactor well only requires 3 milliliters of media to support neuronal organoid growth (Qian *et al.*, 2016). Wang et al.'s organ-on-a-chip technology (described above) was able to maintain organoid cultures at a perfusion rate of 25 μL of growth media per hour (Wang *et al.*, 2018). As an alternative to bioreactors with intrinsic mass transport support, researchers are also utilizing orbital shakers and shaking incubators to promote diffusion of media into organoid cultures (Lancaster and Knoblich, 2014a).

In addition to microfluidic platforms and specialized bioreactors, high throughput generation of organoids is also becoming more feasible due to the increased interest of lab supply manufacturers that are beginning to develop and commercialize 3D cultureware. For example, STEMCELL Technologies, Inc. offers a 6-well plate that can produce an estimated 42,000 EBs at one time (STEMCELL Technologies, Inc., 2020). They also offer neuronal organoid differentiation kits that allow researchers to efficiently mix all necessary growth media components. Given the large number of growth factors and small molecules that must be precisely aliquoted and added to the organoid media at various maturation time points, these differentiation kits offer a mechanism to streamline media production. MIMETAS, B.V.'s OrganoPlate® is a commercially-available microfluidic platform for perfusion culture of ECM-

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encapsulated 3D tissues, including organoids. The OrganoPlate® enables *in situ* gelation of cell-laden ECM and can support up to 96 pump-free perfusion cultures (MIMETAS, B.V., 2020). It has successfully been used to culture 3D gels containing both neurons and glial cells (Wevers *et al.*, 2016). While such 96-well technology may not be of sufficient density to support high throughput screening, such platforms could aid in secondary pharmacology profiling efforts supporting SAR ROPs. Finally, liquid handlers capable of generating, as well as maintaining, 3D cultures are being developed. For example, FORMULATRIX, Inc.'s MANTIS® is capable of encapsulating stem cells in biological hydrogels, and performing media changes without disturbing these 3D cultures (FORMULATRIX, Inc., 2020). Conventional industrial liquid handlers can, however, be used for injection of synthetic hydrogels (as opposed to biological hydrogels) under shear-flow conditions due to their physical properties (Worthington *et al.*, 2017).

The final barrier to the implementation of neuronal organoids in drug discovery is the current lack of assays to measure organoid physiology and function in a high throughput fashion. Without compatible screening assays of complementary throughput, generation of standardized organoids, at-scale, for high or even moderate throughput compound evaluation is not worthwhile. Unfortunately, while the intrinsic three-dimensionality of organoids is crucial to their improved physiological relevance, it is currently unfeasible to adapt many conventional 2D screening assays to this 3D platform; organoids more closely resemble tissue than cells in a monolayer. Poor diffusion of antibodies into the organoid tissue prevents the use of standard immunocytochemical techniques, while light scattering and/or absorption, due to organoid thickness, necessitates altered sample preparation. Visualization of proteins within the organoid interior requires low throughput immunohistochemical techniques such as tissue sectioning

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followed by 2D microscopy or tissue clearing followed by 3D microscopy. Both tissue sectioning and tissue clearing demand time-consuming manual intervention while 3D microscopy necessitates long acquisition times, image analysis with high computational demands, and large data sets (Booij *et al.*, 2019). Due to the inaccessibility of the organoid interior, functional readouts such as electrophysiology and calcium imaging have typically been performed in slices or dissociated organoid cultures rather than in whole organoids (Mariani *et al.*, 2015; Pasca *et al.*, 2015; Qian *et al.*, 2016). Such analyses do not take advantage of the cytoarchitecture of the organoids, and the implications of dissociating organoid tissue are unclear. Finally, extended treatment times required for compounds to enter intact organoid structures and exert their effects must also be taken into account during such functional assay development (Booij *et al.*, 2019).

Emerging microfluidic, imaging, and electrophysiological platforms may be the solution to enabling the high throughput assessment of intact neuronal organoids (Table 3). Along with generating and maintaining organoids, researchers have shown that microfluidic and organ-on-a-chip technologies are capable of *in situ* characterization of organoid physiology. For example, Zhang et al. incorporated real-time biosensors into their microfluidic platform that measured the concentration of soluble proteins as well as imaged the morphology of maturing organoids over days *in vitro* (Zhang *et al.*, 2017). For biomarker detection, they functionalized gold electrodes with antibodies to capture and measure relevant antigens, such as albumin. These electrodes could be multiplexed to measure several biomarkers at a time, and also could be regenerated with fresh antibodies upon saturation with antigen (Zhang *et al.*, 2017). Such integrated electrochemical biosensors could be used for automated compound profiling in organoids with the caveat that, thus far, they have been limited to the detection of secreted proteins.

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Electrophysiological platforms also hold promise for throughput assessment of organoid function. Several researchers have already shown that neither dissociation nor slicing of organoids is necessary to record from organoid neurons. Whole-cell patch clamp recordings performed by Mariani et al. demonstrated equivalent current amplitudes between neurons located in the exterior of retinal organoids and neurons in dissociated retinal organoid cultures (Mariani *et al.*, 2015). Likewise, Quadrato et al. were able to record extracellularly in neuronal organoids using high density silicon electrodes (Quadrato *et al.*, 2017). In this instance, the electrode shank was inserted into the intact organoid and enabled assessment of spike rate and neuronal network dynamics without the need to dissociate and/or slice the cultures. While neither of these examples inherently allows for increased throughput assessment, they provide proof of concept for extracellular recordings and for recordings in whole organoids.

Such findings support the use of multi-electrode arrays (MEAs) for throughput evaluation of organoid function in intact organoids via extracellular recordings. Kathuria et al. used ECM coatings to attach 6 month old cerebral organoids to a 24-well MEA plate, after which they were able to record spontaneous neuronal activity as well as firing rate (Kathuria *et al.*, 2020). Furthermore, MEA technology is commercially available in platforms such as AXION BioSystem, Inc.'s Maestro Pro. This system allows for multiple recordings over months within 96-well plates and provides analysis software to calculate firing rate, neuronal synchronization, and network oscillations within organoids (AXION BioSystems, Inc., 2020).

In addition to microfluidic and electrophysiological platforms, several emerging technologies are increasing the plausibility of analyzing organoids using imaging techniques. In terms of histological techniques, Nagamato-Combs et al. used a mold to embed 8 whole mouse brains in gelatin for simultaneous sectioning on the cryostat (Nagamoto-Combs *et al.*, 2016). If

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followed by immunohistochemistry (IHC) on an automated IHC staining platform (Leica Biosystems, Inc., 2020) and imaged using an automated slide scanner, the application of this technique to organoids could significantly reduce the time and bench work associated with conventional IHC processes.

Tissue clearing is a relatively common histological method that enables immunohistochemical labeling and imaging of 3D specimens without the need for tissue sectioning (Chung *et al.*, 2013). This method involves removing lipids from a 3D specimen to enable penetration of antibodies into otherwise inaccessible tissue. Once the tissue has been cleared and labeled, refractive index matching is performed to allow light penetration into the sample during imaging. While tissue-clearing typically involves time-consuming incubation steps, several automated clearing platforms have recently been commercially developed to streamline this process. These platforms, such as Logos Biosystems, Inc.'s X-CLARITY™ system, can process multiple specimens at a time and often utilize electrophoresis to accelerate lipid removal (Logos Biosystems, Inc., 2019). Similarly, Chen et al. built a microfluidic platform to rapidly clear and label 3D spheroids. Rather than electrophoresis, their system utilizes pressure-driven flow to overcome the limitations of passive diffusion and is capable of boosting fluid exchange by 567-fold (Chen *et al.*, 2016).

Tissue clearing, as well as other emerging tech and protocol adaptations, enables high throughput image acquisition of intact organoid models using conventional high content confocal microscopes. While such platforms are capable of imaging 3D structures several millimeters in thickness, light scattering limits the visualization of the inner tissue beyond a depth of ~50-100 µm within unprocessed tissue. For example, Boutin et al. used high content microscopy to image both cleared and uncleared spheroids in a 384 well plate, and reported superior image quality in

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the cleared spheroids (Boutin *et al.*, 2018). As an alternative to laborious tissue clearing, Durens et al. instead used cell culture inserts to limit organoid growth to 100 µm and enable high content imaging in 96 well plates (Durens *et al.*, 2020). Similarly, Nickels et al. reduced organoid cell density in order to allow for antibody penetration within intact organoids (Nickels *et al.*, 2020). However, as with dissociation of organoids, the downstream consequences of altering physical properties on organoid physiological relevance are unknown. Finally, the emerging technique of "expansion microscopy" uses swellable hydrogels to expand tissue isotropically. Chen et al. showed that this technique is capable of generating transparent specimens for improved light penetration (Chen *et al.*, 2015). While cell density reduction and expansion microscopy have not yet been used in conjunction with whole mount organoid staining followed by high content imaging, such methods could potentially be employed in the future to enable image acquisition using high content microscopy, as well as the other microscopy techniques discussed later.

Sample compatibility aside, several high content confocal microscopes also have software to facilitate rapid image acquisition of 3D structures. For example, PerkinElmer, Inc.'s high content software supports a low magnification pre-scan to locate spheroids and/or organoids within a well, followed by a high magnification scan of only the organoid area to reduce acquisition times (PerkinElmer, Inc., 2020). On the other hand, high content software capable of single-cell segmentation in 3D cultures is still in it's infancy (Booij *et al.*, 2019). Existing software is open-source rather than integrated, and many researchers are choosing to develop their own 3D analysis software (Booij *et al.*, 2019). For example, Boutin et al. developed an internal nuclear segmentation script in order to accurately identify subpopulations of fluorescently-labeled cells (Boutin *et al.*, 2018).

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Due to minimal light bleaching and improved light penetration, fluorescent light sheet microscopy (FLSM) has recently emerged as a popular alternative to confocal microscopy for imaging cleared 3D specimens. While typically low throughput, Eismann et al. recently utilized FLSM for simultaneous imaging of groups of 38 spheroids in 3D. They captured 260 slices at 0.5 µm intervals for each spheroid, and were able to image all 38 individual organoids with these parameters in five minutes (Eismann *et al.*, 2020). Although their spheroids were not thick enough to necessitate tissue clearing, this technique could theoretically be adapted to cleared neuronal organoids. It should also be noted that imaging 228 spheroids in this fashion at five minute intervals over 24 hours generated 10.06 TB of raw data (Eismann *et al.*, 2020). Such large data volumes are not uncommon in 3D microscopy. In order to decrease data usage and maintain throughput, it has been suggested that analysis pipelines in the future utilize automated, real-time phenotypic analyses on low magnification images followed by automated higher magnification acquisition of areas of interest (Booij *et al.*, 2019). Automated recognition of the area-of-interest may be necessary to enable such a workflow.

Imaging platforms that avoid the need for tissue sectioning and clearing of 3D models also exist. Multiphoton microscopy technology allows light to penetrate into thick tissues without sample preparation and is therefore ideal to use with cell-type specific fluorescent reporters to assess proteins of interest. As with FLSM, multiphoton imaging is conventionally low throughput and upright multiphoton microscopes are often incompatible with multi-well plates. However, Rausch et al. recently developed an inverted two-photon microscope for compound screening with the inverted lens promoting sample accessibility within 24-well plates. Using this set-up, they were able to image 40 spheroids per hour in multi-well plates (Rausch and Peker, 2020).

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Aside from microfluidic, electrophysiological, and imaging platforms, it has been shown that several assays conventionally used for high throughput examination of 2D cultures are amenable to use with 3D organoids. For example, Nzou et al. successfully measured ATP production as a proxy for cell viability and metabolic activity within intact neuronal organoids. This assay was performed in a 96-well plate using Promega's CellTiter-Glo® Luminescent Cell Viability Assay with luminescent readouts taken using a standard microplate luminometer (Nzou *et al.*, 2018). Likewise, Vergara et al. used a 3D automated reporter quantification (3D-ARQ) platform to measure oxidative stress and mitochondrial membrane potential using standard assays in whole retinal organoids within 96-well plates. The 3D-ARQ platform necessitates the use of a plate reader that can focus in the z-direction and uses background subtraction of the signal generated by a ubiquitous fluorescent reporter to normalize for organoid size. Vergara et al. estimated that this platform could be utilized to assess over 200,000 samples per day (Vergara *et al.*, 2017). Such technology could also be used with reporters that look at the expression of specific proteins relevant to the neurological condition over time (Vergara *et al.*, 2017).

Although the superior physiological relevance of neuronal organoids has generated a lot of interest in using these models for compound screening in drug discovery, high variability, low throughput generation, and lack of readouts with compatible throughput has limited their utility in drug discovery. Nevertheless, automated technologies are increasingly addressing these hurdles to neuronal organoid implementation in drug discovery. Specifically, organoid production via microfluidic platforms, next generation liquid handlers, and specialized bioreactors represent steps towards miniaturization for higher throughput standardized generation, reduced costs, and the need for minimal manual bench work. Likewise, growth restriction, careful regulation of differentiation lineage, and controlled application of biological

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and mechanical cues has reduced both size and cell type variability between organoids. Finally, integrated biosensors, MEA electrophysiological platforms, automated histology, and high content microscopy hold promise for sufficient throughput evaluation of neuronal organoid physiology and function.

Despite the significant progress that has been made, the generation and evaluation of reproducible organoids on the order required for routine use in early drug discovery may not be readily achievable in the near future. Regardless, the knowledge gleaned from these efforts could still inform the use of slightly less complex 3D models in the context of drug discovery. For example, if the size and heterogeneous nature of neuronal organoids prove prohibitive, the innovative technologies and assays developed to support increased throughput neuronal organoid production and assessment could be adapted for use with neuronal spheroids. The smaller size of spheroids, as well as their less involved fabrication protocol, could potentially make them more amenable for use in compound screening, assuming the desirable attributes achieved in neuronal organoids can be recapitulated in the spheroid model. Furthermore, as neuronal organoid models continue to progress, allowing for their more broad application, researchers should be mindful not to presume an exaggerated value of these models in neuronal drug discovery just yet; there is currently little evidence to support that they are superior to conventional *in vitro* models in their ability to predict clinical compound success, with regard to endpoint achievement. To this end, retrospective screens comparing the performance of successful (or unsuccessful) clinical compounds in neuronal organoids versus conventional *in vitro* models would be an ideal method to evaluate the predictive power of neuronal organoids (Booij *et al.*, 2019). Such an evaluation could help indicate if the potential of neuronal organoids for drug discovery is truly worthy of the further investment in the technology, etc. required to enable their use.

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a. This work received no external funding.

b. This material was not previously presented elsewhere.

c. The authors declare there is no conflict of interest.

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Figures Legends:

Figure 1: Neuronal Organoid Model and Generation Protocol. (A) Neuronal organoids and spheroids are two of the most common 3D *in vitro* models, and they represent an alternative to conventional 2D monolayer cultures. While both neuronal organoids and spheroids contain multiple cell types, organoids are much larger in size, and they develop organized cell layers representative of neurodevelopmental structures. Spheroids, on the other hand, are typically less than 700 µm in diameter and demonstrate a homogenous cytoarchitecture. (B) The standard steps for generating neuronal organoids include: (1) culture of stem cells, (2) aggregation of stem cells into embryoid bodies, (3) encapsulation of embryoid bodies within an extracellular matrix, (4) long term culture with shaking/spinning for improved mass transport. Throughout these steps, the organoid media is supplemented with small molecules and/or growth factors in phases to promote differentiation towards the appropriate neuronal lineage.

Figure 2: Incorporation of Neuronal Organoids into the Hit Identification, Lead Identification and Lead Optimization Efforts. Assuming identified gaps to implementation are bridged, neuronal organoids could potentially be incorporated into several different stages of early drug discovery, including hit ID, LID, and LO efforts. Organoids could serve as a theoretically more physiologically relevant platform to identify meaningful HTS hits. Within the SAR ROP, organoids could be utilized as an alternative, or orthogonal assay, to 2D cell-based assays in order to assess on target-activity. Finally, organoids could be incorporated toward the bottom of the ROP to help facilitate selection of appropriate preclinical candidates. Implementation of organoids at the various phases (arrows) would have unique requirements for at-scale generation and automation, due to the number of compounds being tested.

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Tables:

Table 1: Potential approaches to improve neuronal organoid reproducibility

Solution	Potential Application	References
Patterned micro-wells	Improve consistency in embryoid body size	Ungrin et al., 2008
Physical growth restriction	Improve consistency in organoid size	Ao et al., 2020; Zhu et al., 2017
Restriction of cell lineage fate	Reduce tissue heterogeneity	Qian et al., 2016; Schuldiner et al., 2001; Monzel et al., 2017
3D bioprinting	Improve organoid cytoarchitecture consistency	Cui et al., 2012; Hinton et al., 2015; Jang et al., 2018; Silva et al., 2019
Spatiotemporally defined differentiation cues	Improve organoid cytoarchitecture consistency	Karzbrun & Reiner, 2019; Lancaster et al., 2017; Mahoney & Saltzman, 2001; Silva et al., 2019; Sun et al., 2018; Uzel et al., 2016; Kloxin et al., 2009; Raeber et al., 2005; Przybyla et al., 2016; Yoshikawa et al., 2011
Bioreactors	Improve environmental consistency	Quadrato et al., 2017
Purchase of ECM / media components in bulk	Improve media consistency	
Differentiation protocols using only small molecules	Improve media consistency	Reinhardt et al., 2013
Synthetic hydrogels	Improve ECM consistency	Langhans, 2018

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Table 2: Potential methods to enable higher throughput neuronal organoid generation

Solution	Potential Application	References
Microfluidics	Automation of embryoid body seeding, media changes, and encapsulation with ECM	Ao et al., 2020; Y. Wang et al., 2018; Wevers et al. 2016; MIMETAS, B.V.
3D liquid handlers	Automation of embryoid body seeding, media changes, and encapsulation with ECM	FORMULATRIX, Inc.
Bioreactors	Miniaturization for reduced media consumption, cost, and footprint	Qian et al., 2016; Y. Wang et al., 2018
Differentiation kits	Streamlined media production	STEMCELL Technologies, Inc.

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Table 3: Potential techniques for higher throughput assessment of neuronal organoids

Solution	Potential Application	References
Microfluidics	Automated biosensing	Y. S. Zhang et al., 2017
Multielectrode arrays	Automated measurement of electrophysiological properties	Kathuria et al., 2020; AXION BioSystems, Inc.
Gelatin embedding en masse	Throughput sample preparation prior to imaging	Nagamoto-Combs et al., 2016
Automated immunohistochemistry	Throughput sample preparation prior to imaging	Leica Biosystems, Inc.
Automated tissue clearing	Throughput sample preparation prior to imaging	Y. Y. Chen et al., 2016; Logos Biosystems, Inc.
Restriction of organoid size and/or density	Throughput sample preparation prior to imaging	Durens et al., 2020; Nickels et al., 2020
Confocal high content microscopy	Throughput analysis of histological markers and probes	Boutin et al., 2018
Fluorescent light sheet microscopy	Throughput analysis of histological markers and probes	Eismann et al., 2020
Multiphoton microscopy	Throughput analysis of histological markers and probes	Rausch & Peker, 2020
Microplate readers	Throughput analysis of histological markers and probes	Vergara et al., 2017; Nzou et al., 2018

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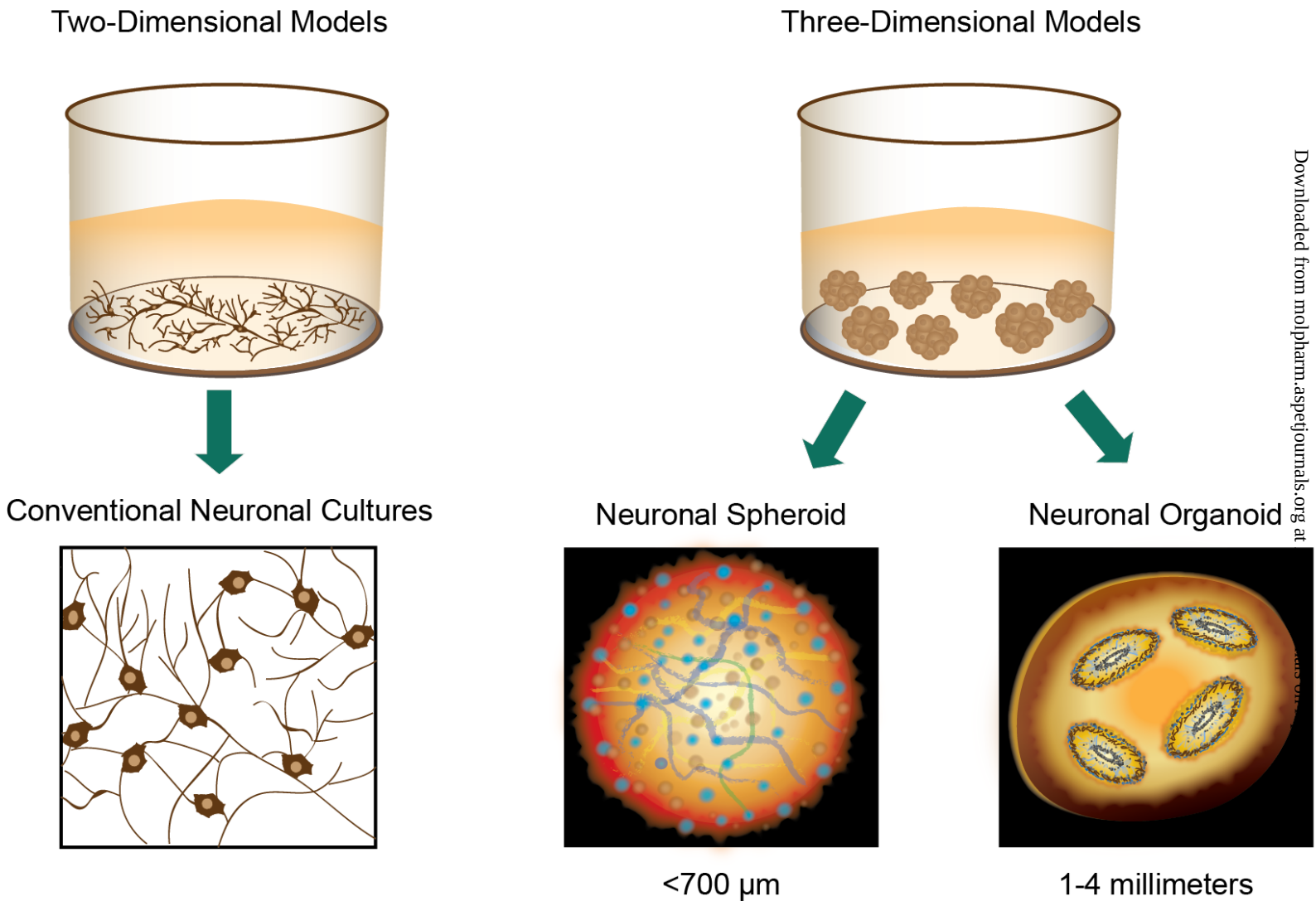

Figure 1A

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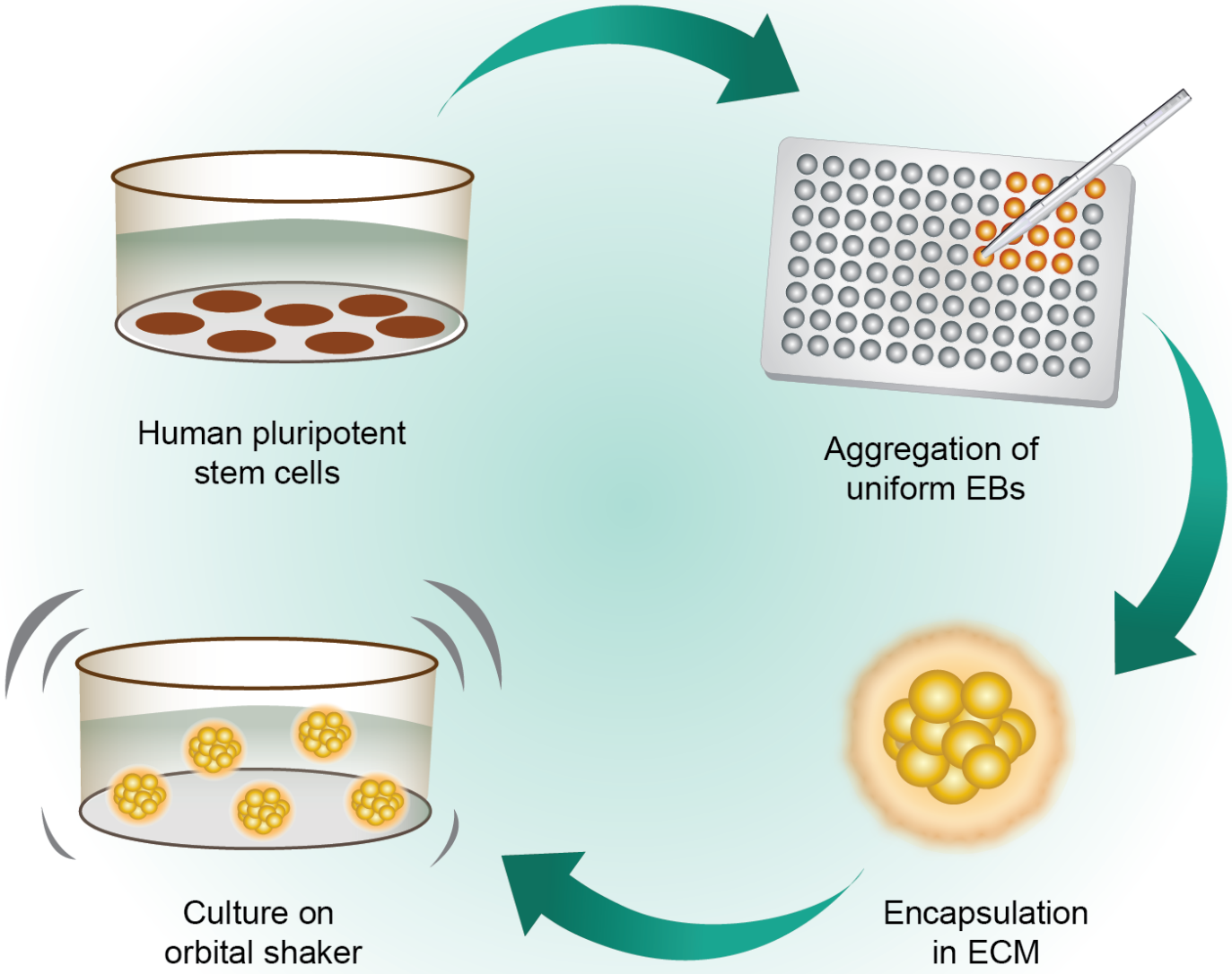

Figure 1B

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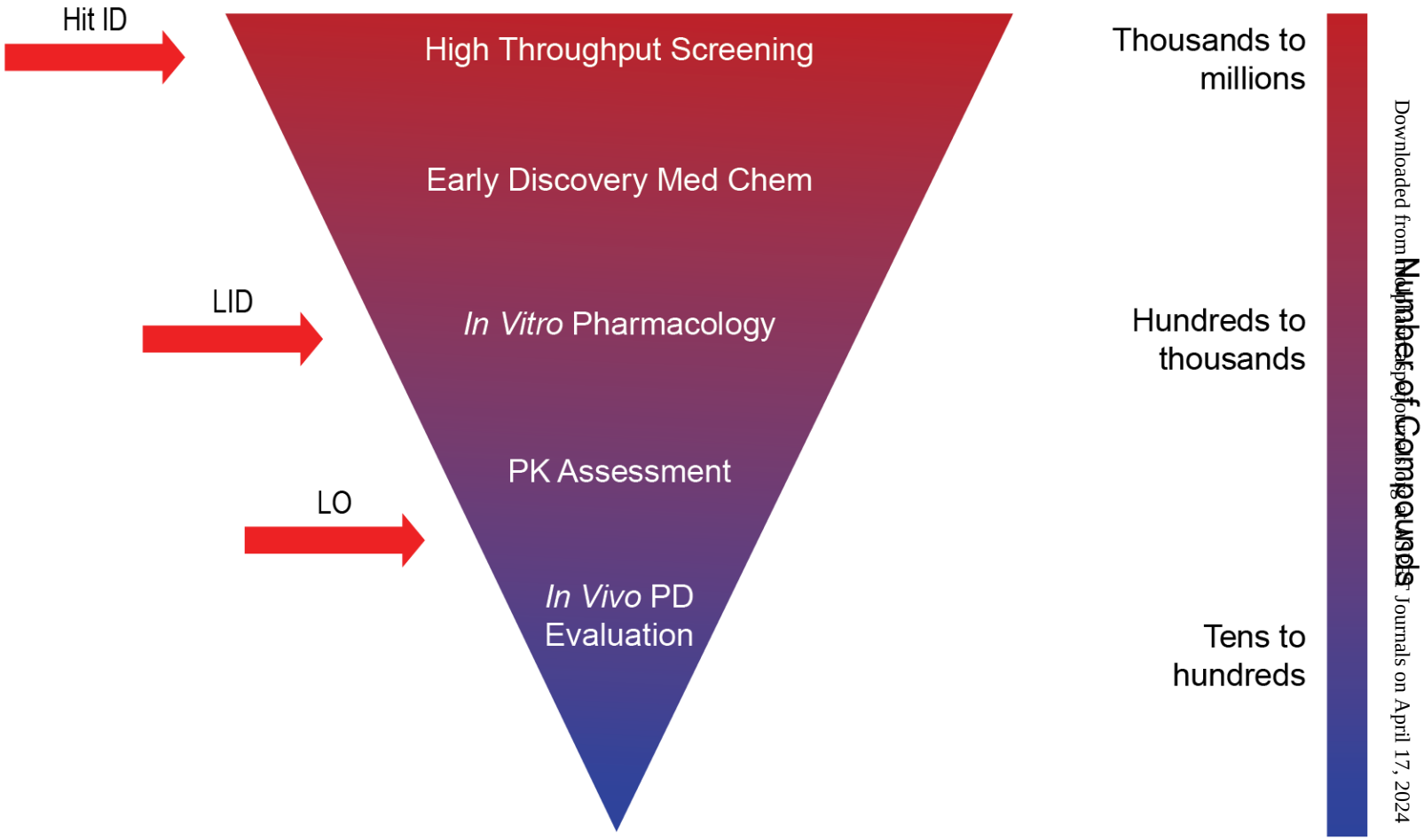

Figure 2

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