WHITEPAPER

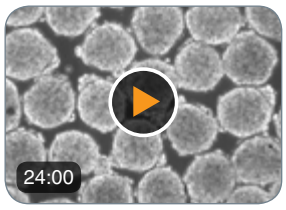
Aggrewell™ Plates for Drug Discovery and Regenerative Medicine Applications

Uniform and Scalable EB Formation for Drug Discovery and Regenerative Medicine Applications

The developmental versatility of pluripotent stem cells (PSCs) offers a powerful approach for directing cell fate, and these cells are a promising source of progenitors and terminally differentiated somatic cells for cell replacement therapy and drug screening applications.[1] In culture, PSCs have a unique trait of unlimited propagation while retaining their inherent potential to differentiate into cells from the three embryonic germ layers. When plated onto a non-adherent culture dish, these cells aggregate to form embryoid bodies (EBs) which undergo a process of spontaneous differentiation that closely imitates early human embryogenesis.[2]

Three-dimensional EBs derived from PSCs better mimic physiological tissues[2], and can therefore, capture the cellular functions and responses that are functionally relevant. As a result, biological research findings and drug assays based on EBs offer greater predictive capability in comparison to conventional two-dimensional cell culture systems. Consequently, EBs can be used as a model system to not only study the interaction of cells with their microenvironment, but also utilize them for investigating potential compound toxicity in embryos and somatic cell populations.[3]

The development of pharmaceutical drugs entails screening compound libraries using in vitro models to identify candidate molecules that would go into animal studies and further clinical trials. Cell-based in vitro assays are a key component of drug discovery research as they provide predictions of drug metabolism and toxicity in vivo. Typical in vitro models do not truly recapitulate the in vivo physiological environment, as the cells are confined in two-dimensional mono-layers without the key physical and chemical cues which underlie their identity and function in vivo.[4] In this regard, the importance of 3-D structures, such as EBs, is self-evident as they better mimic physiological tissues and create growth conditions that more precisely mimic the in vivo behavior of cells. Further, refinement of the in vitro environment significantly influences the manner in which cells respond to small molecules. However, scaling-up and maintaining reproducibility of processes for EB generation have been difficult due to lack of standardized tools and processes that are both simple and scalable.



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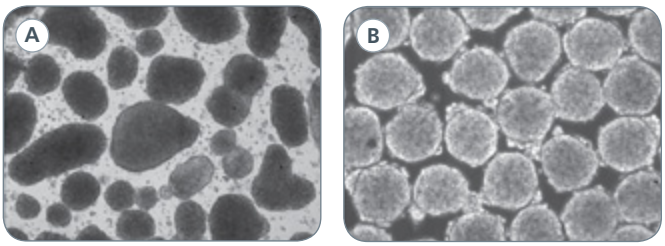
Embryoid Bodies generated using AggreWell™ Plates are:

- Uniform in size and shape
- Reproducible
- Size-controlled

Aggrewell™-based technology solves this issue by aggregating PSCs into EBs of a defined size, using microwells, and thereby allows adoption of EBs into routine use in early research and pharmaceutical development. Aggrewell™ plates provide a means to generate a large population of EBs of uniform size and shape (Figure 1B). By controlling the size of EBs, researchers have added control over differentiation outcome and are better able to standardize differentiation protocols, while the AggreWell™ plates scalability helps to increase cellular throughputs.

FIGURE 1. Generate uniformly sized embryoid bodies using AggreWell™.



(A) Human EBs formed using conventional methods are heterogeneous in size and shape. (B) Human EBs formed using AggreWell™ plates for 24 hours are uniform in size and consistently spherical in shape. Shown are EBs generated with 2,000 cells using AggreWell™400. Photos taken at 100x magnification.

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Traditional EB Formation Methods

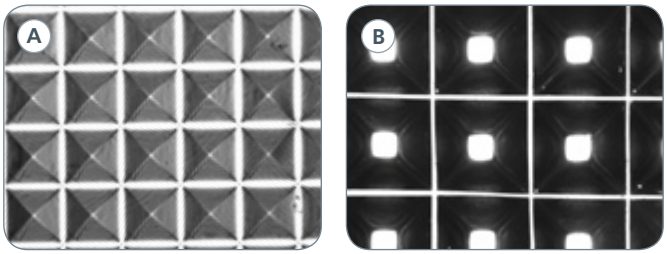
Conventional EB formation methods result in EBs that are heterogeneous in size and morphology, leading to inefficient and uncontrolled differentiation (Figure 1A). These methods are inherently incapable of large-scale production and exhibit limited control over cell aggregation during the EB formation process.

One of the traditional EB formation methods involves cultivation of suspended PSCs in hanging drops underneath a petri-dish lid. The process entails inverting the lid subsequent to positioning the drops. As a result, the drops are susceptible to disruption, falling, dispersion or merging with their neighbors. While the technique is economical, it is labor-intensive, does not allow mass production, and is incompatible with high-throughput screening. In addition, media-exchange is complicated as it requires an additional step to transfer the EBs manually to another culture dish for long-term maintenance and further experiments.

Other conventional EB formation techniques include static suspension culture, triangle-bottomed 96-well plates, microcontact printing, and non-destructive laser-based methods. Recently, new devices have been developed to improve EB formation and their further manipulation and analysis. For instance, biocompatible coating materials and specialized hanging drop plates have been designed to induce and control the overall process. These methods also have their inherent disadvantages, such as variability in EB morphology including size and shape, and long-term culture. It is also difficult to scale up these processes in a high-throughput manner for generating end-stage, terminally differentiated cells in clinically relevant numbers and for small molecule screening.

STEMCELL Technologies' Aggrewell™ plates are a novel, standardized platform for EB production. These EBs can serve as experimental tools for generating somatic cell types, studying developmental biology and screening small molecules. The technology entails centrifugal forced aggregation to generate uniform, size-controlled EBs. The process is scalable in a high-throughput manner and offers superior control of EB size without requiring complex handling procedures.[5]

FIGURE 2. AggreWell™ contains microwells to make uniform cell aggregates.

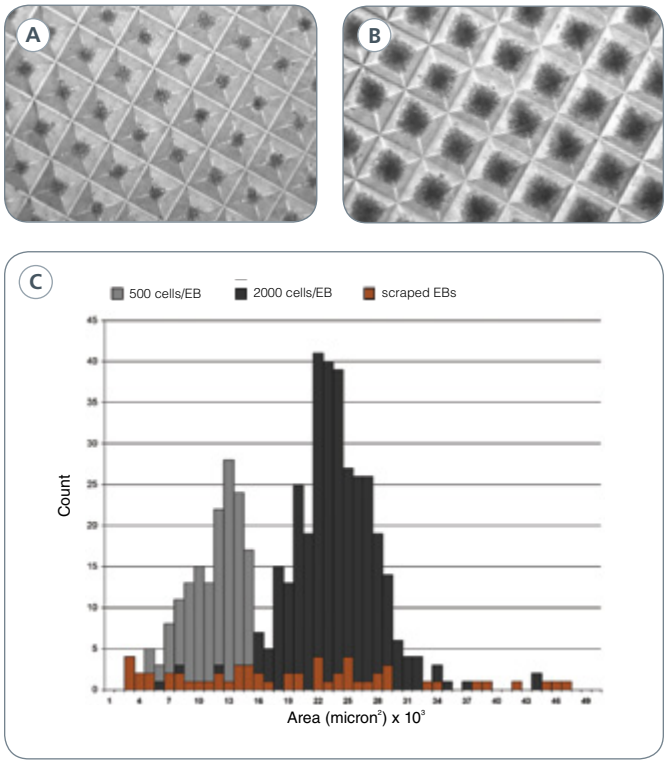


(A) AggreWell™400 and AggreWell™400Ex plates contain microwells 400 µm in diameter. Photo taken at 40x magnification. (B) AggreWell™800 plates contain microwells 800 µm in diameter. Photo taken at 40x magnification.

Reproducible Production of Uniformly-Sized Embryoid Bodies using Aggrewell™ Plates

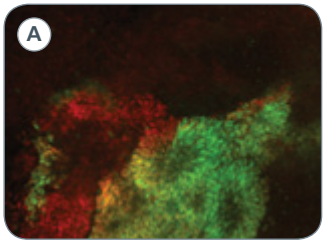
AggreWell™400 and AggreWell™400Ex plates have microwells 400 µm in diameter, and AggreWell™800 plates have microwells 800 µm in diameter (Figure 2). EB formation is accomplished by adding a single cell suspension to the plate, centrifuging to distribute the cells evenly among the microwells, and then culturing for a minimum of 24 hours to allow aggregation of the cells within each microwell. The resulting EBs are highly uniform in size (Figure 3) and can be efficiently differentiated into a variety of cell types (Figure 4). AggreWell™ plates bring an easy and standardized approach to the production of EBs, making further experiments more reproducible. EBs and other cell aggregates generated using AggreWell™ plates are consistent in size and shape, and uniform within and between experiments.

FIGURE 3. EB size is controlled by adjusting cell density in AggreWell™.

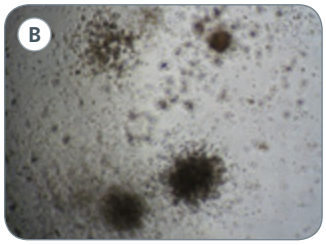


The size of EBs can easily be adjusted using AggreWell™ plates. EBs were formed by seeding the wells of an AggreWell™400 plate with varying concentrations of single hES cells so that approximately (A) 500 or (B) 2,000 cells seeded each microwell. (C) The size of EBs can be tightly controlled using AggreWell™400 plates (light grey & dark grey bars), unlike scraping protocols which give a wider distribution of sizes (red bars). Size determination was done with ImageJ software.

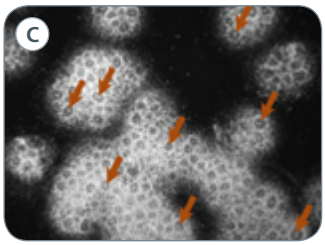
FIGURE 4. EBs generated using AggreWell™400 plates are able to differentiate into multiple cell types.



Neural rosettes stained with PAX6 (green) and SOX2 (red).



Hematopoietic cells were detected using CFC assays.



Differentiated cells were generated by culturing AggreWell™400 - generated EBs in serum-containing suspension culture for (A) 4 days (neural) or (B) 14 days (hematopoietic)*. Neural cells were assayed by plating onto gelatin-coated dishes, and hematopoietic cells were assayed by plating in MethoCult™ (Catalog #04434). (C) 100% Neural Induction can be achieved using AggreWell™ 800 plates and STEMdiff™ Neural Induction Medium (Catalog #05831). Neural aggregates of 10,000 cells each were formed in an AggreWell™800 plate and STEMdiff™ Neural Induction Medium and cultured for 5 days with daily ¾ volume medium changes. Neural aggregates were then harvested and plated onto PLO/L coated plates. Attached neural aggregates are shown 2 days after attachment with prominent neural rosette structures visible (arrows). Magnification 20x.

*Data reprinted from Ungrin et al., 2008.[5,8] See reference for full culture details.

Uniformly-Sized EBs as a Tool for Toxicology Assessment Studies

The PSC-derived EB model has been introduced to overcome the limitations of cytotoxicity assays for the determination of toxic effects of drugs in vitro. Human pluripotent stem cells (hPSCs) differentiated in EBs consist of progenitor cells of all three germ layers: ectoderm, mesoderm and endoderm. The rationale behind the EB model is that early developmental processes simulated by EB differentiation are affected by treatment of undifferentiated PSCs with toxic substances and as a consequence, specific differentiation processes are inhibited, induced or accelerated[6]. The altered gene expression profiles induced by exposure to toxic compounds could be a direct effect on specific molecular pathways. Alternatively, these changes may reflect altered levels of differentiation into different cell types comprising the EB.

For instance, a potent human teratogen, retinoic acid (RA), when applied at different concentrations and specific time intervals of EB differentiation, affects the differentiation capacity of mouse embryonic stem cells (mESCs) in a concentration and time-dependent manner. hPSC-derived EBs also mimic the teratogenic phenotype when exposed to RA by exhibiting alterations in neural-specific genes. They show changes in the molecular phenotype with specific up-regulation of anterior HOX genes. Significant deviation from normal development process of EBs is seen which is evident by increase in HOXA1 and NCAM1 expression.[7]

Lithium Chloride has similar distinguishable time- and development-specific effects on mESC differentiation. mESC-derived EBs when exposed to lithium chloride at high concentration cause reduction in cardiac α-MHC and skeletal muscle-specific MyoD, but slight increase in neural-specific synaptophysin and NFM transcript levels and therefore, leads to inhibition of cardiac and myogenic differentiation.[8]

Increased expression of endodermal markers is observed in EBs derived from hPSCs when exposed to ethanol, another known teratogen. This suggests increased differentiation propensity of the cells towards definitive endoderm.[7] In mouse EBs, ethanol triggers apoptotic pathways during in vitro differentiation of mESCs to EBs. Further, exposure to ethanol down regulates SSEA-1 and therefore, affects the regulation of transcription factors during differentiation.[9]

The above examples highlight the value of EB-based screening models in identifying potential teratogenic compounds and appropriately assessing their effect on early developmental processes as well as cellular differentiation.

Also, the EB-based model system can be used to provide data on the reversal of a compound-induced developmental toxicity. For instance, arsenic at non-cytotoxic concentrations significantly down-regulates expression of genes representative for all germ layers. Elimination of this arsenic induced dysfunctioning was observed by treating EBs with a newly developed arsenic antidote, Monoisoamyl Dimercaptosuccinic Acid (MiADMSA). MiADMSA treatment reduces arsenic induced visceral and skeletal defects in rats.[10] Therefore, in addition to studying the effects of compounds, the EB model may also be useful to identify compounds that can revert or prevent the effects of certain toxic compounds.

The recent developments in the Aggrewell™ technology (Aggrewell™400Ex) have underlined our efforts to implement a novel, scalable strategy for compound testing based on PSC generated EBs. Uniform and homogenous EBs generated through Aggrewell™ plates can be used for the analysis of tissue-specific genes via reporter gene expression. They can be applied as a high-throughput screening platform to evaluate changes in gene and protein expression patterns on exposure to different compounds. Their application can assist in identifying endpoints suitable for determination of embryotoxicity in differentiating PSCs, and such proof-of concept in vitro systems may be useful to predict human-specific developmental toxicity.

Most of the above effects can be recapitulated in induced pluripotent stem cell (iPSC) derived EBs. In addition to possessing similar pluripotent capabilities, these cells hold the benefit of originating from individuals with a particular phenotype and genotype. This facilitates application of directed human sub-population models early in drug screening and toxicology assessment studies.

Therefore, EBs produced under strictly controlled conditions using Aggrewell™ plates provide a robust experimental tool to screen potential teratogenic compounds, thereby complementing existing in vivo and in vitro models.

Uniformly-Sized EBs as a Tool for Generating Terminally Differentiated Somatic Cells

EB formation from PSCs is a common method for producing cells of different lineages for further applications. Generating such terminally differentiated cell types from PSCs using EBs is well documented. The efficiency with which this method can differentiate PSCs into different cell types is highly variable and it is difficult to produce pure (> 90%) populations of cells. This is due to both the heterogeneous morphology of EBs that are generated using conventional methods, and the non-directed (often serum-containing) media conditions used. Therefore, these methods lead to inefficient and uncontrolled differentiation.[5] In this regard, a significant challenge is the ability to consistently produce EBs and further differentiated cells in the quantity, quality, and purity required for both therapeutic and screening applications.

As described earlier, Aggrewell™ technology is a well defined, standardized, scalable production platform to reproducibly produce uniform sized EBs. Such EBs can be used for controlled large-scale generation of terminally differentiated cell types for clinical as well as industrial applications. EBs produced using Aggrewell™ plates can be size-controlled (Figure 3) as the EB size influences the final density of terminally differentiated cells types from PSCs. Uniformity in EB size and morphology is significant for reproducibility of differentiation. The Aggrewell™ plates can be seeded with various cell numbers to readily generate different sized aggregates.

Studies have revealed that controlling EB size in a directed cardiac differentiation system regulates cardiogenesis from human embryonic stem cells (hESCs) by influencing the probability of an EB to generate cardiomyocytes and the corresponding density of cardiomyocytes relative to other cell types in a differentiating EB.[11] This can be measured by the percentage of EBs contracting as well as the number of cardiomyocytes by flow cytometry. The probability of a smaller EB (100 µm microwell) undergoing cardiogenesis is lower than that of a larger EB (300 µm microwell). However, smaller EBs develop cardiomyocytes at a much greater density. Such size control provides an important parameter to increase the overall efficiency of cardiomyocyte differentiation process. The importance of EB size in regulating cardiac differentiation is associated to diffusion of essential substrates as well as the role of various cues via growth factors. Most techniques use signaling molecules (Activin A, BMP4, bFGF, and Wnt) to direct differentiation of hESCs to cardiomyocytes. Combination of Aggrewell™ technology to regulate the size of EBs with controlled growth factor delivery in a defined microenvironment can provide a powerful tool in the reproducible differentiation of PSCs to cardiomyocytes.

Aggrewell™ plates have also been employed to expand and differentiate hPSCs towards the definitive endoderm lineage. A significant improvement in yield and scalability has been demonstrated in the overall differentiation process together with reduced experimental variability. Uniform, size-controlled, cellular aggregates held in individual microwells of the plate on exposure to specific differentiation cues give rise to cells positive for C-KIT and CXCR4, characteristic markers of endoderm. A significant up-regulation in SOX17, FOXA2, Cerberus, and GATA3 expression is also seen. The cells derived are capable for subsequent pancreatic and hepatic differentiation. Such an integrated expansion and differentiation system based on Aggrewell™ plates has the capacity to generate endoderm progenitors in clinically-relevant numbers required to support pre-clinical studies.[12]

Generation of uniform homogenous and size-controlled EBs can assist hPSC-derived, end-stage, terminally differentiated cells to be implemented into routine toxicology assessment studies. Aggrewell™ plates allow establishment of reliable and reproducible EB production and differentiation procedures, which can be also be performed in a high-throughput format. In such format, the predictability, sensitivity and specificity of the respective test systems can be demonstrated for a wider panel of drugs and/or compounds. Furthermore, EBs allow distinguishing the capacity of human specific toxic effects from the corresponding mouse cells and assist in recognizing species-specific differences by performing comparisons between both systems.

Conclusion

Aggrewell™-based EB generation is a versatile and simple-to-use technology for both life science research and drug screening applications. The technology has the potential to vastly simplify and streamline EB formation for EB-based assays to produce reliable results.

We believe PSC-derived EBs provide a new approach to prospectively screen compounds for possible toxic effect which can either complement or replace existing in vivo and in vitro models.

The introduction of fully-defined media including mTeSR™1 and TeSR™2 that allow robust, straight-forward hPSC expansion and maintenance under feeder-free conditions (and derivation of hESC/hiPSC lines) promise to promote faster adoption of such a system in embryotoxicity testing and small molecule screening. In summary, we anticipate that hPSCs can be manipulated to generate reproducible models based on EB formation for screening of potential toxicants. Further, the system may also contribute to our better understanding of molecular composition of embryo microenvironment.

For more information about AggreWell™, email us at aggrewell@stemcell.com or visit www.stemcell.com/aggrewell.

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