UNIVERSITY OF CALIFORNIA, SAN DIEGO

Single-Cell Neurosphere Assay: A Method to Quantify Neural Stem Cells Clonogenicity on a Single Cell Level

A Thesis submitted in partial satisfaction of the requirements for the degree

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Biology

by

Givon Sanati

Committee in charge:

Professor Alexander Groisman, Chair
Professor James Kadonaga Co-Chair
Professor Randy Hampton

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The thesis of Givon Sanati is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

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Dedication

I dedicate this thesis to Katayoun and Jamshid Sanati for their love and support.

Table of Contents

List of Figures

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ABSTRACT OF THE THESIS

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The progression of stem cell therapy continues to be interrupted by the absence of consistent stem cell clonogenicity protocols. Previously administered clonogenicity assays demonstrate some promise; however, they are restricted to specific cell types. We take advantage of a microfabricated approach to study neural stem cell clonogenicity on a single cell level. We engineer a microarray platform using silicone matrix technology to study clonogenicity. In addition, we use image cytometry to quantify functional characterization of clonogenicity. Lastly, we explore and compare known stem cell biomarkers to further validate our functional assay. We found that early passaged neural

stem cells demonstrate higher neurosphere formation capacity as compared with later passaged neural stem cells. To further confirm this outcome, we identified CD133 as a known clonogenic biomarker for neural stem cells. We determined a possible correlational relationship between CD133 and neural stem cell clonogenicity. We establish and confirmed a novel approach to study clonogenicity. This approach will allow for a more reliable method to compare and study stem cell potency.

Introduction

The advancement of stem cell therapy continues to be interrupted by the absence of consistent stem cell characterization protocols (Pastrana et. al 2011). Recent reports have demonstrated the potential of stem cell therapy by demonstrating the ability to replenish damaged and aging tissues (Warren. Et. Al 2009). Nevertheless, stem cell therapies gradual progress may have resulted from improper characterization (Burdon et. al 2011). Stem cell characterization remains as a crucial factor for the progression of stem cell therapy. Subsequently, numerous assays have continued to reveal modest characterization. We used a microfabricated approach as a potential solution.

To elucidate this problem, we begin to describe the brief history of behavioral characterization assays. Biomarkers (SOX2, BMI1, CD133, CD117, etc.) are the primary tool to assess stem cell quality (Molofsky et. al 2003; Ying et. al 2008). This approach has been problematic for the absence of analysis in a behavioral setting. Thus, assays to study stem cell behaviors are applied to confirm a relationship with the biomarkers and to provide a more valid conclusion of stem cell quality (Reynolds et. al 2005). A principal method for studying stem cell behavior is utilizing its ability to produce clones (Sarugaser et al. 2009). T.T. Puck and Philip I. Marcus published this first practical and efficient method for growing colonies from individual animal cells, and would refer to this assay as the clonogenic assay. (Puck et. al 1956). “This assay involves three key steps: (1) The treatment is administered to a sample of cells; (2) The cells are seeded in a tissue culture vessel and allowed to expand; (3) The colonies formed are analyzed” (Franken et. al 2006; Franken et. al 2006; Puck et. al 1956). Clonogenicity assays can be used to study the effectiveness of specific agents to prevent the growth of a cell into a

colony (Hoffman et. al 1991; Franken et. al 2006; Reya et. al 2001). To look at a more cost effective and time efficient approach, we focus on *in vitro* clonogenicity assays.

To expand on the current behavioral characterization protocols, we next discuss adherent clonogenicity assays and their possible limitations. A common and inexpensive *in vitro* assay for determining clonogenicity is the colony formation unit assay (CFU). This assay requires the culturing of cells at low seeding density over a short period of time (Vertelov et. al 2013). Conversely, this approach restricts the cells to a two-dimensional platform, and thus misrepresents the cells proliferation potential. This problem may compromise the physiological relevance of the assay (Pastrana et. al 2011). Mesenchymal stem cells are a great candidate for this application because of their biological niche (Hoch et. al 2014). Nonetheless, other cell types, including neural stem and hematopoietic stem cell, thrive in a three-dimensional setting. Other approaches such as the three-dimensional neural colony formation assay (N-CFCA) offer a solution for this problem (Azari et. al 2011). In the N-CFCA, stem cells are seeded at high density in a viscous collagen-based platform for colony formation. Conversely, stem cells experience an impairment of typical growth rate. This problem arises from the low permeability of the viscous collagen based semi-solid. As a result, growth factor diffusion is impaired to the cells in culture (Pastrana et. al 2011). Another important drawback is the high seeding density of the N-CFCA. This detriment represents the clonogenicity of a cluster of cells rather than each individual cell (Pastrana et. al 2011; Azari et. al 2011). Adherent clonogenicity assays have demonstrated limitations for certain cell types. Hence, we next discuss non-adherent clonogenicity assays (Pastrana et. al 2011; Azari et. al 2011).

A common non-adherent clongenicity assay is the sphere formation assay. Cells are dissociated and suspended in culture to form colonies in the shape of spheres (Ahmed et. al 2009). Cell density is based on the cell type. Yet, this parameter becomes controversial because cell density may have a direct influence on clonogenicity (Pastrana et. al 2011). Previous groups have based clonogenicity on number and sizes of the spheres (Coles-Takabe et. al 2008; Azari et. al 2011). Nonetheless, the occasional formation of irregular aggregates are produced. This problem is due to the cells proximity and highly dynamic structures. Also, this may be the result of the intrinsic and spontaneous locomotion of the cell while suspended in culture (Chen et. al 2005; Pastrana et. al 2011). This can lead to an inaccurate representation of the stem cell potency. These irregularities demand the utilization of an innovative system utilizing microscale technology. This will enhance reliability and validity, thus developing a more robust clonogenic assay to study stem cell behavior.

To address this challenge, a microfabricated approach was administered to help improve this problem. Microfabrication has transformed the study of cell biology by engineering microenvironments essential for various cell culture applications (Liu et. al 2013; Lee et. al 2013; Harink et. al 2013; Song et. al 2009). These platforms have allowed for the development of intricate microenvironment to understand stem cell behaviors on a micro-scale level (Pastrana et. al 2011). Consequently, an established and robust system is critical to perform long-term cultures of stem cells in these micro-fabricated niches (Chang et. al 2015).

In this study, we take advantage of microfabrication by designing and modeling a micro-fabricated device to improve the study of clonogenicity on a single cell level. Using microengineering technology, we generate an abundance of microwells over a small surface area of a standard 24 well plate. We optimized the surface chemistry of the base membrane using a combination of polydimethylsiloxane (PDMS) polymer and polyethyl glycol acrylate (PEG-Acryl) macromers. To further test our design, we culture neural stem cells and examine colony formation efficiency on a single cell level. Finally, we compare neural stem cell biomarkers with neurosphere formation efficiency to confirm a more valid conclusion of stem cell potency.

Materials and Method

Expansion of neural stem cells

Stemedica cell technologies provided human fetal NSCs (P0 NSCs) that was previously dissociated from fetal brain tissue. Theses P0 NSCs were thawed and re-suspended in NSC media. Cell were seeded at 20,000 cells/cm2 on a 100 mm tissue culture dish (Corning®, Corning, NY) for P1 passage for 10 days with media change every 2-3 days. On day 10, cells were harvested, dissociated and re-seed as previously mentioned. NSCs were passaged up to four passages (P4) and upon each day of harvest a certain number of cells were frozen away for further analysis. NSC media composed of 1X B27 (LifeTechnologies, NY, USA), 1X N2 (LifeTechnologies, NY, USA), fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), epithelial growth factor (PeproTech, Rocky Hill, NJ, USA), and (D)MEM/F12+Glutamax™-I basal medium (LifeTechnologies, NY, USA). The hypoxia culturing was performed in a humidified 5% O2 and 5% CO2 incubator at 37°C.

Marker Expression

Cells were stained with appropriate antibodies conjugated to R-Phycoerythrin (PE) (Miltenyi, San Diego, CA, USA) and expression scored by flow cytometry using standard protocols. The antibodies used were SOX2, Nestin, CD19, CD34, CD11b, CD133, HLA-DR and isotype control, diluted according to the manufacturers' recommendations.

Differentiation

For astrocyte differentiation, cells were seeded at high density and allowed to grow to confluence. Before the culture was replaced with differentiation media, separate culture was stained with Nestin antibody (Covance, San Diego, CA) as a positive control. The media was removed and replaced with appropriate differentiation media (Life Technologies, San Diego, CA). They were cultured further for two weeks with intermittent media changes. Astrocytes were distinguished and stained with GFAP antibody (Stem Cell Technologies, San Diego, CA) for immunohistochemistry.

Fabrication of the Microwell Array

Micropattern silicon wafer was provided by MuWells for PDMS microwell casting. Polydimethylsiloxane microwells were formed using Sylgard 184 Silicone Elastomer Kit (Ellsworth Adhesives Germantown, WI) with the addition of 0.1 % Poly(ethylene glycol) methyl ether methacrylate (Sigma Aldrich, St. Louis, MO) to make the precursor solution (Fig 1A.). The structure of each microwell was cylindrical. The dimensions were engineered to have a diameter and height of 300 microns by 200 microns with a spacing of 150 microns per microwell (Figure 1).

Microscopy to Asses Patterning and NSC cell culture

Standard stereoscope was used to qualitatively assess the micropatterning process and to determine the dimensions of the microwells. NucBlue® Live ReadyProbes® Reagent (Thermofischer San Diego, CA) was used as a fluorescent stain to monitor cell presence NSC entrapment in the microwells. In addition, neurospheres were stained with

NucBlue for quantification of neurosphere formation efficiency, diameter and size with the Celigo Image Cytometer (Celigo Inc. San Mateo, CA).

Statistical Analysis

Data are representative of three or more independent experiments and presented as mean ± SEM. Statistical significance was assessed using Student's unpaired t-Tests (Microsoft Excel). Probability values of less than 0.05 were considered significant.

Results

Neural Stem Cell Characterization and Differentiation

Through the implementation of flow cytometry, we identified 100% of our P0-P4 NSC populations to be expressing neural stem cell biomarkers SOX2 and Nestin. Cell surface markers CD11b (microglia), CD19 (B cells), CD34 (progenitors/endothelial cells), and HLA-DR were used to analyzed P0-P4 NSC. Flow cytometry analysis revealed no difference in the expression of surface markers between P0-P4 NSCs. The quantity of HLA-DR-positive cells did not surpass 2% expression.

CD133 (Porminin-1) has been previously identified as a NSC clonogenicity marker (Sun et. al 2009; Suslov et. al 2002). We used this biomarker to confirm the NSC colony formation of serially passaged NSCs. The magnitude of the CD133+ population was previously tested in serially passaged NSCs by flow cytometry. Here, we observed a gradual decrease in CD133 expression in NSCs with increased passaging. In the P0-P4 NSCs, a bimodal population was present with a CD133+ population ranging from 2-21% (Fig. 1A). P4 NSCs demonstrated the lowest expression of CD133, whereas P0 expressed the highest. Moreover, we observed a gradual decrease in the CD133 expression per NSC passage. Hence, this marker may be as a suitable candidate to assess clonogenic populations of NSCs on a single cell level. Correspondingly, P0-4 NSCs were all able to differentiate into glial cells when growth factors were removed (Fig. 1B). We observed no variation of NSC P0-P4 in terms of differentiation.

Development of PEG PDMS Microwell Arrays

Microwell arrays were developed by modifying a previous method established for a glioblastoma cancer cell culture (Tkachenko et al., unpublished). Polydimethylsiloxane (PDMS) was used as the primary culture substrate for single cell neural stem cell culture.

Its chemically inert and non-toxic properties allow for an easily molded material to create a micro-fabricated device. A micro-patterned silicon wafer was provided to us by MuWells as a mold for substrate casting. We fabricated micro-well arrays from a combination of polydimethylsiloxane and PEG-acrylate polymers to make the precursor solution. The mixture was poured onto a micropatterned silicon wafer and incubated at 60 ^{o}C for polymerization. The mold was extracted and bonded to the bottom of bottomless 24-well plate after plasma treatment (Fig. 2A). We used stereoscopy to measure the dimensions of the microwells. Each 24-well contained approximately 1200 cylindrical patterned microwells each with a diameter of 300 μm, a depth of 200 μm, and spacing of 150 μm (Fig. 2B). Standard microscopy revealed the two dimensional view of the microwell micropatterns for neural stem cell culture (Fig. 2C). It is important to note that the PDMS-PEG precursor solution completely cross-linked resulting in cylindrical patterned microwells with a negligible depth of only 1.8 ± 0.2 microns.

Neurosphere Formation Efficiency of Neural Stem Cells

To initiate our NSC single cell entrapment, we established a ratio of 1 cell per every 2.5 wells using the Poisson distribution probability theory. We began by monitoring single cell division of individual NSCs. Initially, we retrieved P4 NSCs and counted the number of cells per micro-well for up to 21 days (Fig. 3B). We used the Celigo imaging cytometer to monitor population doubling. We observed the first population doubling to be an average of approximately ~60 hours (data not shown). After 72 hours, approximately 70% of the cells divided and the remaining 30% remain in growth arrest. To assess a single-cell functional relation for neurosphere-formation in NSCs, we compared P0 and P4 NSCs. P0 and P4 NSCs were monitored in culture for 21

days for cell survival. Using the Celigo, cell survival was assessed by recording the number of viable cells on day 0 and day 21. We did not observe a significant difference in cell survival (Fig. 3C). However, we observed a variability base on sphere size using standard microscopy.

To validate this observation, we decided to distinguish the neurospheres in relation to size. Based on the literature and our study, we decided to label a neurosphere as a spheroid of 50 microns or greater. P0 NSCs demonstrated a significantly higher frequency of larger neurospheres as compared with P4 NSCs allowing us to conclude that P0 NSCs have a higher neurosphere formation efficiency (NFE) compared to P4 NSCs (Fig. 3C) ($p<0.01$).

NSC's NFE may decrease after every passage. To asses this hypothesis, we compared P0, P2 and P4 NSCs in terms of NFE, diameter and cell number per neurosphere. P0, P2 and P4 NSCs were in seeded at 1 cell per 2.5 microwell (Poisson distribution) in each well of the 24-well plate. Next, NSCs were entrapped and cultured for 21 days in culture. Again, we used the Celigo to quantify our analysis. NSCs displayed a gradual decrease in NFE, diameter and cell number per neurosphere with increased passages (Figure 4). This phenomenon correlated with the previously characterized biomarker, CD133, and allowed for a stronger reliability of the biomarker. Neural stem cell biomarker, CD133 showed possible correlation with NFE.

Association of CD133 expression and neurosphere formation efficiency

To test the potential for this assay, we compared NSCs obtained from two different donors. P2 and P4 human neural stem cells from two different donors were

provided by Stemedica Cell Technologies, Inc. Using flow cytometry, we observed a higher expression of CD133 in P2 cells when compared with the P4 cells (Fig. 5A, $p<0.01$). Next, we administered the single cell neurosphere assay to study NFE. Once more, we observed a higher NFE in the P2 lots compared to P4 lots (Fig. 5A, $p<0.01$).

To conclude our analysis, we tested five additional P4 lots from five separate donors which were obtained from Stemedica Cell Technologies Inc. to further support the functional representation of our assay. Using flow cytometry, we observed a variable expression of CD133 per P4 lot of different donors. Next, each lot was seeded in microwell plates at 1 cell per 2.5 microwell (Poisson distribution) for NFE. We observed a variable NFE in relation to each lot. This may be a result of slight variations in procedures during isolation, point of development at time of NSC isolation, etc. Here, we suggest a correlational relationship between CD133 expression levels and NFE (Fig. 5B&C). This analysis further demonstrated the possible advantage of our single cell neurosphere-formation assay as well as the enhanced reliability of biomarker characterization.

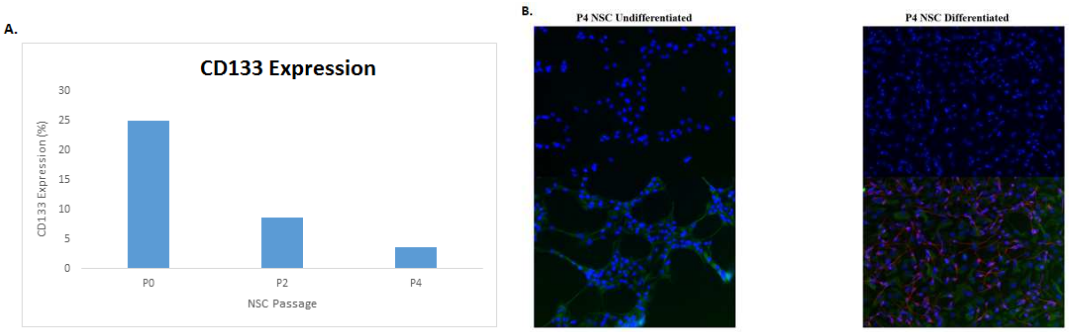


Figure 1. NSC Characterization.

A. Flow cytometry data to analyzed NSC CD133
B. Differentiation data to demonstrated NSC Multipotency.

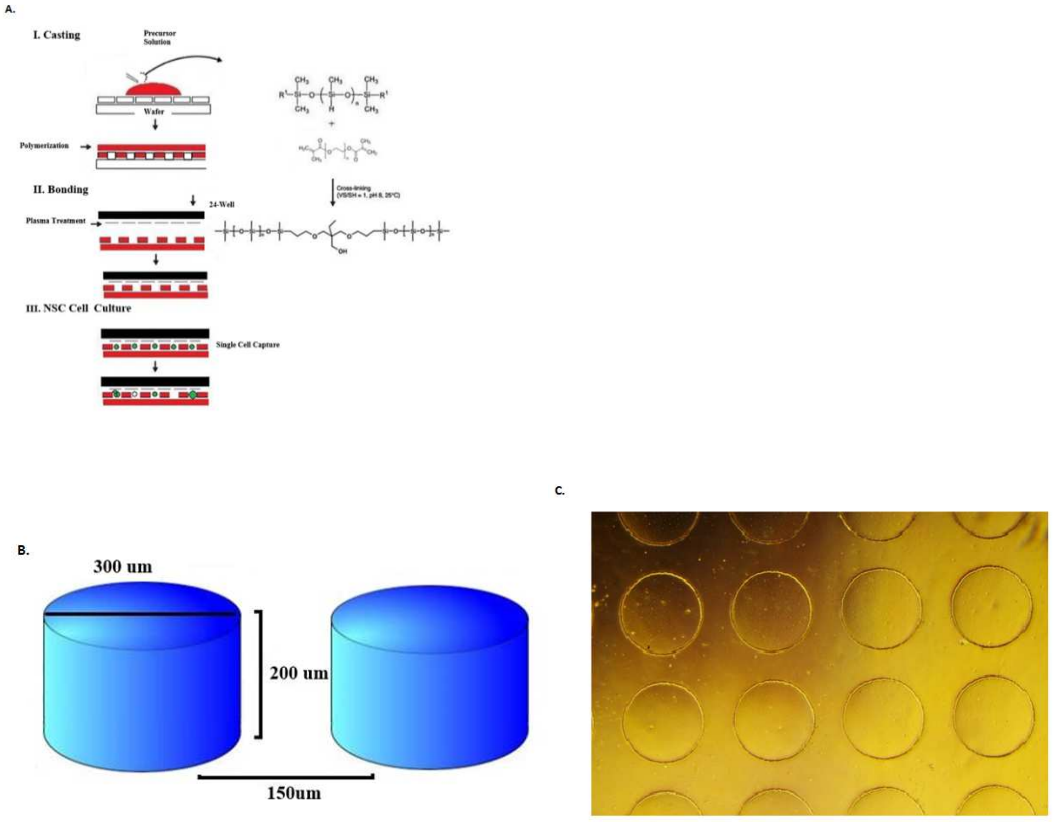


Figure 2. Multistep process to fabricate PDMS microwell arrays for NSC culture.

A. Polydimethysiloxane-Poly(ethylene glyocol acrylate) (PDMS-PEG) are casted on a fabricated micropatterned silicon wafer and incubated at 25^{o} C to polymerize (Step I). A bottomless 24-well plate underwent plasma treatment to create an adhesive surface for attachment (Step II). Microwell arrays are sterilized and sealed for subsequent stem cell culture (Step III).
B. Obtained dimensions of the microwells: 300 ± 2.3 micron diameter, 200 ± 1.8 micron depth and 150 ± 0.7 micron separation between adjacent wells.
C. 2 Dimensional view of the microwells using standard microscope**.**

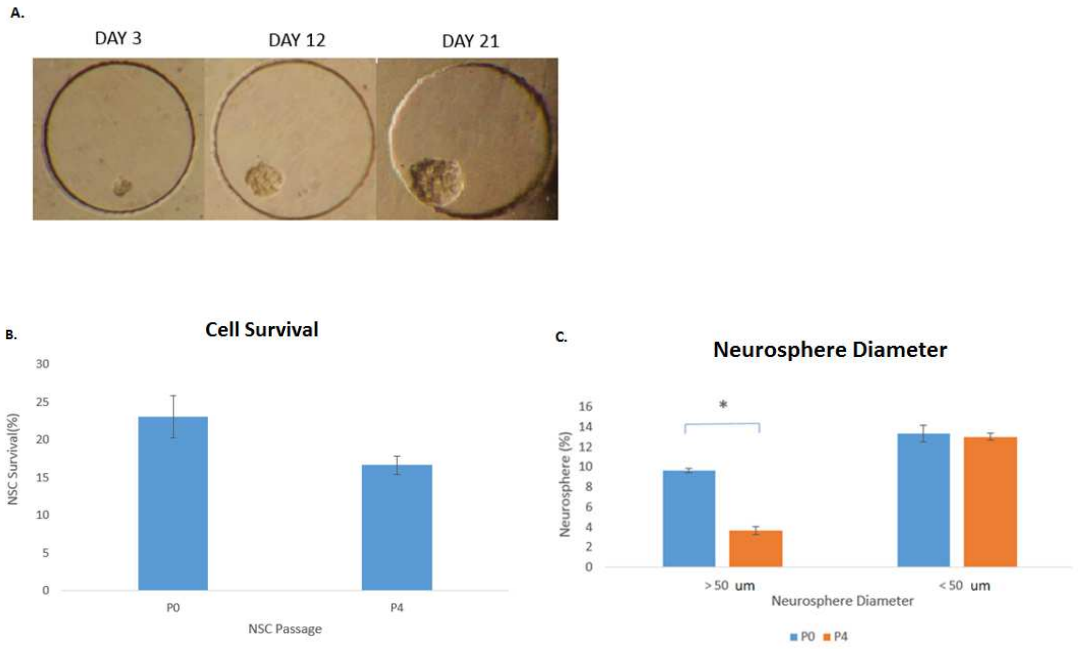


Figure 3. Fate and viability of single neural stem cell (NSC) culture on microarray. In vitro neurosphere formation was assayed on PDMS-PEG microwell plate of (dimensions of 300 micron by 200 micron). Each condition was tested in four wells; each experiment was repeated four times. The bars represent 1 SEM (Standard error of the mean) from four independent experiments combined (n = 16). * - $p < 0.05$.

A. Single P4 NSCs placed into the microwells. Using compound microscopy, the NSCs were visually inspected to determine the time of declined expansion of the neurospheres (NS), which was observed at 21-days of culture.
B. To assess the potency of P4 NSCs, a single-cell viability was assessed using a more "stem" P0 NSCs. After 21-days of culture, single-cell viability was quantified using a live/dead staining kit and Celigo Imaging Cytometer to measure cell sustainability.
C. Further quantification of P0 &P4 NSCs diameter after 21-day culture to determine the minimum size required to be categorized as a NS. P0 NSCs seeded on a single-cell level in the microwell over a 21-day culture displayed a significantly higher number of NS 50 microns or greater.

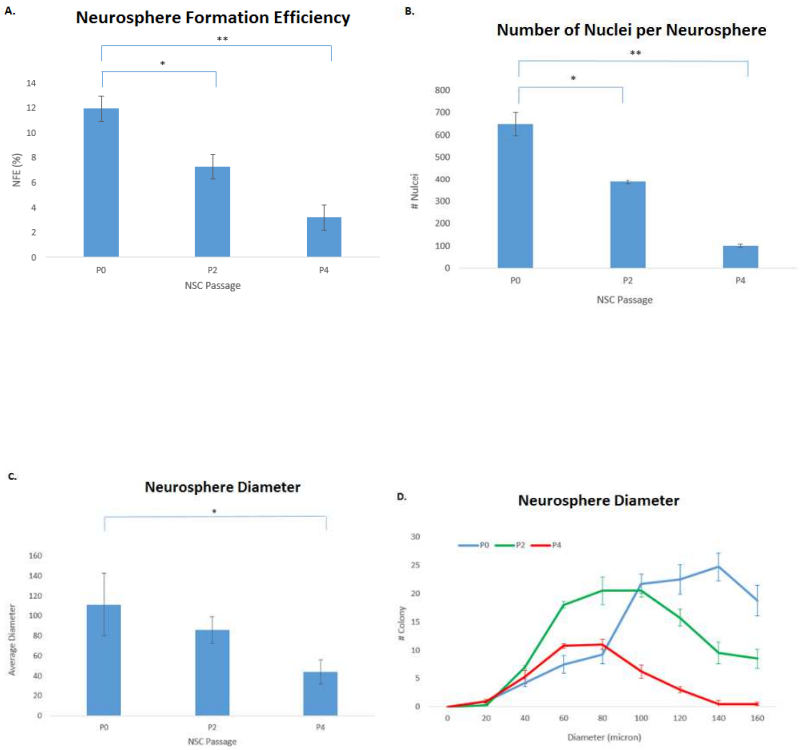
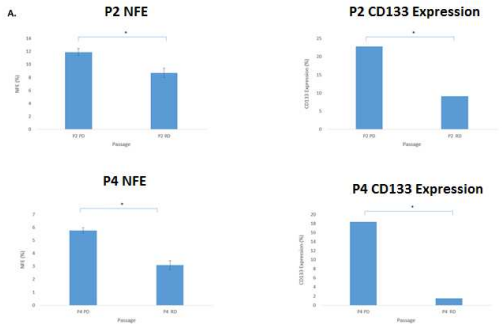
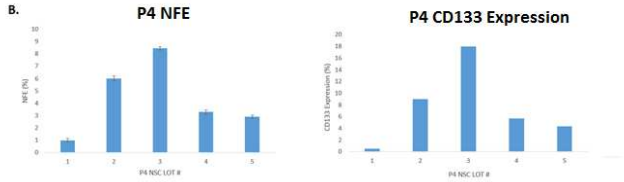


Figure 4. Neurosphere Formation of Serially Passaged NSCs. In vitro neurosphere formation was assayed on previously mention microwell plate to assess serially passaged neural stem cells. Each condition was tested in four wells; each experiment was repeated four times. The bars represent 1 SEM (Standard error of the mean) from four independent experiments combined (n = 16). * - $p < 0.05$, ** - $p < 0.01$.

A. The microwell plates were used to culture serially passaged NSC for 21 days in 5% oxygen to measure neurosphere formation efficiency.
B. The number of nuclei in each neurosphere were quantize to determine the average number of NSCs in the neurospheres in each passage using the Celigo image cytometer
C. The size of the neurospheres were measured using the Celigo image cytometer to determine the average diameter of the serially passaged NSCs.
D. Histogram of neurosphere diameters generated after a 21 day culture period reveals a large heterogeneity in neurosphere sizes. Neurosphere were counted and their area and diameter were measured via image analysis (Celigo) from fluorescent images.





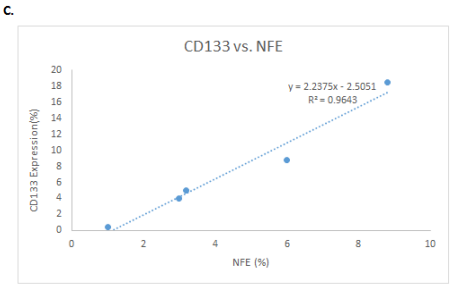


Figure 5. The CD133 correlates with enhanced NFE in neural stem cells. Each condition was tested in four wells. The bars represent 1 SEM. * - p <0.05.

A. The microwell plates were used to culture passage 2 and 4 NSC of two different donors (PD & RD) for 21 days in 5% oxygen to measure neurosphere formation efficiency. Flow cytometry data of CD133 was assessed of passage 2 and 4 (N=12).
B. The microwell plates were used to culture passage 4 NSC of five different donors labeled 1-5 for 21 days in 5% oxygen to measure neurosphere formation efficiency. Flow cytometry data of CD133 was assessed passage 4 NSCs of different donors (N=12).
C. Correlational plot comparing P4 NSC CD133 expression in comparison to neurosphere-formation efficiency

IV.

Discussion

Neural stem cell characterization

Human fetal neural tissue was provided by Stemedica Cell Technologies. Cell were serially passaged in 5% oxygen over a two-month period. Before implementing the micro-fabricated approach to the NSCs, we characterized the cells in terms of biomarker and differentiation potential. We determined the cells of P0-P4 expressed the same level of biomarker expression and differentiation potential. The only biomarker tested that demonstrated variability was CD133. This marker has previously been reported as a clonogenicity marker for NSCs. CD133 was the only marker that demonstrated a gradual decrease per passage. NSCs have a very complex *in vivo* niche. Tampering with this niche may lead to differentiation and loss of potency. CD133 may be a distinctive marker for NSC characterization due to its gradual decrease in expression in *in vitro* culture.

Engineering the optimum micro-well plates for neural stem cell culture

MuWells provided us with a micro-pattern silicon wafer for plate casting. The dimensions of the wells were measured using a standard stereoscope. The material that provided the highest throughput in efficiency was a combination of polydimethylsiloxane (PDMS) and polyethylene glycol to permit a non-adhesive cell suspended single cell culture (Figure 1). We used PDMS as the primary substrate for microwell casting. PDMS is highly hydrophobic and filling the microwells was deemed difficult. Likewise, this chemical property created a semi-adherent environment. This consequence prevented a non-adherent suspended cell culture. We next explored the possibility of reducing this surface tension by adding different polymers to decrease surface tension. We chose 0.1 % polyethylene glycol acrylate (PEG-Acry) because it has low toxicity. This polymer

served as another primary component of our precursor solution to reduce this adhesive property. Subsequently, we were able to develop a micro-fabricated device to study NSC clonogenicity in a suspended single cell culture.

Neural Stem Cells and Neurospheres

To assess the micro-fabricated technology, we examined P4 NSC s to determine clonogenicity. After 30 days in culture, we were able to distinguish a heterogeneous population of NSC spheroidal colonies. After day 21, the NSC colonies did not demonstrate any rapid increase of clonogenicity in terms of number and size. We deduced that 21 days would be the optimum time required to detect and analyze NSC colonies. We then retrieved P0 and P4 NSC to compare clonogenicity of early and late passaged NSCs. First, we compared cell survival of P0 and P4 NSCs during the 21-day culture. We concluded to see no significant difference in cell survival. To our finding we discovered a variability in neurosphere size when comparing P0 and P4 NSCs. Early passaged NSCs demonstrated a significantly higher neurosphere-formation efficiency (NFE). In addition, we further quantified the NFE of P0-P4 NSCs. We measured NFE, size and number of nuclei per neurosphere. There was a significant difference between serially passaged NSCs in terms of NFE, diameter and average number of nuclei per spheroid. We also managed to generate a gradient detailing the distribution of neurosphere size of each NSC passage. This approach allowed for possible validation of the previous hypothesis. It is perceived that serially passaged NSC's NFE decreases while *in vitro* culture.

CD133, the clonogenicity marker

Previously in our NSC characterization, we observed a gradual decrease in CD133 expression after ever passage. Biomarkers are the primary candidate for stem cell potency characterizations. Developing a robust functional assay would help enhance some validation of the biomarkers. Here, we identified CD133 as a possible extracellular marker correlating to NFE in NSCs. Stemedica Cell Technologies Inc. provided us with P2 and P4 NSCs of different donor lots. While comparing passage 2 and 4 cells of different tissue sample lots, we witnessed a greater expression of CD133 in earlier passaged NSCs. In addition, we examined a higher NFE in P2 cells as compared with P4 cells (Figure 5). We further substantiated our hypothesis by retrieving previously passaged P4 NSCs of five different donors and compared this biomarker expression to their NFE. Similarly, we observed an association of CD133 expression with NFE (Figure 5). We conclude a possible correlation of CD133 and NFE. Here, we suggest a more innovative way to certify the behavioral validity of biomarkers.

Looking Forward

To our knowledge, the biology of stem cells and their *in vivo* niche continues to expand. Biomarkers are the primary approach for characterizing stem cells, yet they are not sufficient to determine potency. Behavioral applications must be applied to determine value of the biomarkers. This marks a crucial emphasis to advance new *in vitro* assays that overcome the restrictions and practical downsides of preceding clonogenicity assays. Previously established clonogenicity assays have shown some promise; however, they display many instances of variability. This new assay will assess clonogenicity at a

single cell level. Conversely, this single cell neurosphere assay developed may have some drawbacks. One apparent problem is the incapability to detect quiescent/dormant stem cell. This population of stem cells may play an important role in stem cell delivery. This setback/complication will create an opportunity to explore further advancement of this technology. Engineering culture matrices in the micro-wells may become an important step toward high throughput assays.

Conclusion:

The advancement of stem cell therapy faces many challenges of delivery of potent cell types. Stem cell characterization may play a crucial role in helping understand and improve the delivery. Recent functional assays for capturing primary stem cells ability to proliferate and form clones have come under examination (Doe et. al 2008). Biomarkers are the primary choice to assess stem cell potency. However, there have been some reports of them demonstrating variable potency (Reynolds et. al 2005). This may be due to possibly unexplored examinations of behavioral parameters. To address this problem, we developed a single cell neurosphere formation assay using a microfabrication to study stem cell behavior. We were able to develop a micro-fabricated device using polydimethylsiloxane (PDMS) in combination with a polyethylene glycol acrylate (PEG-acryl) as the proper material to enable a viable NSC culture on a single cell level. Next, we monitored neurosphere formation of early passaged and later passaged NSC to compare neurosphere formation efficiency (NFE). Lastly, we establish a correlational relationship between the extracellular NSC marker CD133 and NFE of the NSC s to

confirm a relationship of biomarkers and function al behaviors. This approach will allow for a more trustworthy approach to compare and study stem cell potency.

V.

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