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High Throughput Isolation and Directed Differentiation of Mesenchymal Stem Cells

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Abstract

Cell based therapies such as bone marrow and islet transplantation provides patients with functional cells to supplement disease compromised cells. One major limitation of cell based therapies is providing adequate numbers of functional cells to alleviate disease symptoms. Mesenchymal stem cells (MSCs) are multipotent progenitor cells used for cell based therapies via bone marrow transplant, and more recently for cardiovascular and neurodegenerative diseases. Since MSCs are multipotent and easy to obtain, the aim of this study is to obtain large amounts of functional MSC derived cells for cell based therapies via high throughput cell isolation and laser guided assembly for directed differentiation of cells. Successful cell therapy requires efficient cell isolation, maximal cell survival, and optimum differentiation environments. Efficient MSC isolation from adipose and bone marrow tissue is obtained through high throughput flow cytometry based cell sorting. After isolation, cells are placed in a polyethylene glycol diacrylate (PEGDA based) hydrogel using arrays of optical traps to place single cells into an array. Mimicking the *in vivo* environment of the cells is important for successful differentiation, and the hydrogel provides a three dimensional scaffold that allows the cell to rest in a matrix that is more like a tissue microenvironment than a liquid culture dish. Seeded in the hydrogel along with the cells are microspheres holding reagents that are used to stimulate lineage specific induction of MSCs. The microspheres are thermally excited to release the contents in a controlled and time dependent manner, thus leading to control differentiation events. Placement of microspheres and concentration of reagents affect differentiation outcome and differentiation is observed in the hydrogel by confocal microscopy and cell surface markers. Currently, the laser guided systems and microsphere placement are being optimized using U937 cells. Using high throughput flow cytometry in conjunction with optical trapping we plan to study directed differentiation of stem cells *in vitro* and provide large numbers of functional mesenchymal derived cells for cell based therapies.

Introduction

Mesenchymal stem cell (MSC) therapies have the promise of treating several diseases such as cardiovascular and neurodegerative diseases. A rate limiting step for the clinical use of mesenchymal stem cell therapies is efficiently obtaining and isolating a viable, homogeneous population of stem cells as well as targeted differentiation into specific lineages. To investigate these issues, we are using two newly developed technologies: the iCyt HAPS high throughput flow sorter (**Table A**) and laser guided trapping of cells[1]. Before working with trapping and differentiating MSCs, we are optimizing laser trapping and differentiation conditions. We selected U937 cells as a model for MSCs. U937 is a human cell line established from a diffuse histiocytic lymphoma that displays many monocytic characteristics, can be easily differentiated in culture, endure severe culture conditions, and be induced easily (72 hrs) to differentiate into several cell types depending on the molecular signal. During differentiation, U 937 cells undergo a phenotypic change from small, non adherent cells to clumping, adherent cells that are easily to visualized using nitroblue tetrazolium (NBT) reduction assays, making it a suitable model for this study[2]. U937 cells are trapped in a polyethylene glycol diacrylate (PEGDA based) hydrogel and surrounded by an array of microcapsules containing differentiation reagents, such as phorbol 12 myristate 13 acetate (PMA). The microcapsules are manipulated to release reagents in a controlled manner, thus allowing us to control the time and amount of differentiation reagents released. The system developed here can be used to further study monocyte/macrophage differentiation and environmental interactions. After optimization with U937 cells, we plan to continue work with MSCs derived from porcine and human cord blood, adipose, and bone marrow tissues in the same fashion as the U937 cells. At the conclusion of this study, we will establish methodologies for high throughput sorting of stem cells as well as trapping and differentiating cells in a 3D scaffold for applications such as cell based therapies.

Objectives

- To establish high throughput methods to rapidly sort pig and human stem cells using iCyt technologies
- To laser assemble U 937 and MSCs in hydrogel without loss of viability or differentiation capabilities
- To effectively differentiate cells in hydrogel along specific lineages in a time controlled manner using microspheres
- To establish a viable model and methodologies to trap U937 and MSCs in a 3D hydrogel scaffold

Materials and Methods

- U937 Cells were cultured in RPMI 1640 Media and passaged every 2- 3days
- For differentiation, cells were cultured with phorbol 12 myristate 13 acetate (PMA; Sigma) at a concentration of 20, 50, or 100 nM for 24 or 48 hours. Differentiation was assessed by visual changes including increased size, culture dish adherence and cell clumping as well as nitroblue tetrazolium assays (NBT; Sigma). RAW 264.7 cells were used as a positive control and undifferentiated U937 cells were used as a negative control.
- Cells were trapped in 10% to 20% polyethylene glycol diacrylate (PEGDA) hydrogel with 0.01% 2 hydroxy 2 methylpropiophenone (Photo initiator). Viability assays were conducted using Molecular Probes live/dead viability/cytotoxicity assay.
- U937 cells were trapped in a 4x4 array using a Zeiss Neo fluor 100X, 1.25NA objective at λ=900nm with a time averaged power per trap at the sample of 8mW/trap.

References

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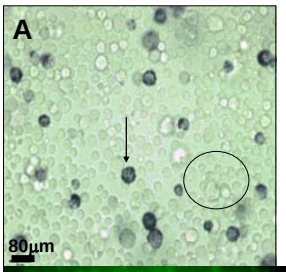
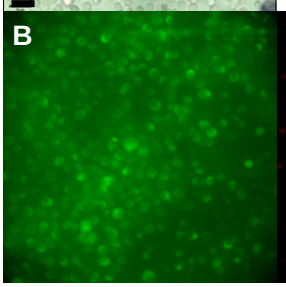
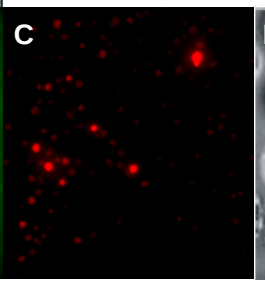
Results

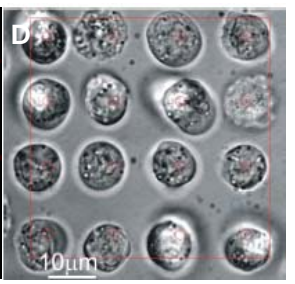
U937 cells readily undergo differentiation into macrophages. U937 cells were induced to differentiate with the addition of PMA at 20nM, 50nM and 100nM for 24 or 48 hours. After removal of PMA (72 to 96 hours), a NBT assay was performed. Fully differentiated cells (**Panel A**) reduced NBT and appear blue in color (arrow). Other differentiation characteristics include larger size and adherence. A cluster of undifferentiated cells is shown in the circle. U 937 cells readily underwent differentiation after adding PMA at each concentration tested, but lower concentrations of PMA (20mM) and shorter incubation times (24 hours) resulted in fewer cells reducing NBT. The differentiated phenotype of NBT reduction and larger size and adherence was readily lost. However, the highest concentration of PMA (100nM) and longest incubation time (48hrs) led to an increase in NBT reduction, and the differentiated phenotype persisted for over one week after removal of PMA. In hydrogel, PMA assays will determine the lowest concentration of PMA and shortest incubation time leading to differentiation of single cells. These culture differentiation experiments provide a starting point for hydrogel assays. (10x magnification, scale bar 80 μm).

Viability Assays. In order to determine viability of U937 cells in hydrogel, we preformed the Molecular clones live/dead assay. Traditional viability assays such as trypan blue exclusion and flow cytometry cannot be used in hydrogel, as trypan blue cannot penetrate the hydrogel and cells cannot be removed from the hydrogel for flow cytometry. The live/dead kit stains live cells with Calcein AM (green, **Panel B**) and dead cells with Ethidium homodimer 1(red, **Panel C**). In this figure, cells were trapped in 20% hydrogel and 0.2% photo initiator. The live/dead assay was performed 24 hours after trapping. Maximum viability tests in hydrogel are still being performed. We have observed that a range of 10 20% PEGDA with 0.1% photo initiator resulted in a higher number of viable cells. (10x magnification).









U937 Cells can be effectively directed into a hydrogel array. To demonstrate and establish methodologies for directing cells into a 3D scaffold, U937 cells were manipulated into an array using newly developed laser trapping techniques (**Panel D**). Briefly, cells were trapped in a PEGDA hydrogel with 0.1% photo initiator using a Zeiss Neo-Fluor 100X, 1.25NA objective at λ=900nm with a time-averaged power per trap at the sample of 8mW/trap. Successful trapping into this 4x4 array shows that U937 cells can be effectively trapped in a PEGDA-based 3D scaffold with laser capture techniques. Next, viability (Molecular Probes live/dead) assays are being conducted on trapped U937 cells followed by hydrogel placement and PMA differentiation in hydrogel.

Ongoing Research

U937 cells are being used as a proof of concept experiment before continuing on to stem cells. Currently, our lab is sequencing and developing antibodies for porcine CD34 to use for isolation of HSCs and MSCs. Layton et al. recently developed antibodies for porcine CD34, which has previously been unavailable[3]. These antibodies will then be used with the iCyt Highly Animated Parallel (HAPS) module to sort cells. The HAPS modules are enclosed in a biosafety cabinet and offer faster sorting times than a traditional flow sorter (**Table A**). The highest speed traditional cell sorter would have the throughput of a single HAPS module. The development of HAPS technology provides the same multi color measurement capabilities as FACS, allowing both analysis and sorting to be scaled as needed. Because this system was designed with clinical cell separations in mind it will translate more easily to a clinical setting than traditional FACS systems. After establishing isolation procedures, MSCs will be placed in hydrogel in the same matter to the U937 cells to produce a living 3D MSC array. Differentiation of MSCs along specific lineages will be monitored by phenotypic changes and cell markers. Upon successful establishment of methodologies to trap of porcine stem cells in a 3D scaffold, we then plan to manipulate human MSCs.

Table A

Sort Parameters	1 Haps Module	4 Haps Modules	16 HAPS Modules
Maximum Cell Analysis Rate (Cells per second)	100,000	400,000	1,600,000
Maximum Cell Sorting Rate (Cells per Second)	75,000	300,000	1,200,000

Conclusions

- U937 cells are able to differentiate under normal culture conditions. Cells maintained the differentiated phenotype of NBT reduction, large size, and adherence best when differentiated with 100nM PMA for 48 hours.
- Cells are able to withstand hydrogel conditions for at least 24 hours
- U937 cells can be manipulated into arrays
- Methodologies for placement of cells into a 3D scaffold are being established

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