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Title: Differentiation of Embryonic Stem Cells into Cardiomyocytes in a Compliant Microfluidic System

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Abbreviated Title: Cardiogenesis in Compliant Microfluidic Devices

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Abstract

The differentiation process of murine embryonic stem cells into cardiomyocytes was investigated with a compliant microfluidic platform which allows for versatile cell seeding arrangements, optical observation access, long term cell viability, and programmable uniaxial cyclic stretch. Specifically, two environmental cues were examined with this platform – culture dimensions and uniaxial cyclic stretch. First, the cardiomyogenic differentiation process, assessed by a GFP reporter driven by the α-MHC promoter, was enhanced in microfluidic devices compared with conventional well-plates. The addition of BMP-2 neutralizing antibody reduced the enhancement observed in the microfluidic devices and the addition of exogenous BMP-2 augmented the cardiomyogenic differentiation in well plates. Second, 24 hours of uniaxial cyclic stretch at 1Hz and 10% strain on day 9 of differentiation was found to have a negative impact on cardiomyogenic differentiation. This microfluidic platform builds upon an existing design and extends its capability to test cellular responses to mechanical strain. It provides capabilities not found in other systems for studying differentiation, such as seeding embryoid bodies in 2D or 3D in combination with cyclic strain. This study demonstrates that the microfluidic system contributes to enhanced cardiomyogenic differentiation and may be a superior platform compared with conventional well plates. In addition to studying the effect of cyclic stretch on cardiomyogenic differentiation, this compliant platform can also be applied to investigate other biological mechanisms.

Key Terms: uniaxial cyclic stretch, cardiogenesis, embryoid bodies, bone morphogenetic protein 2, stem cell therapy

Introduction

Microfluidic devices (μFDs) are excellent *in vitro* systems in which to study cell functions, build disease/organ models, and dissect mechanisms of specific stimulations in a systematic manner.[19] Previous work from our laboratory has demonstrated that μFDs can be used to examine interactions of multiple cell types and effects of chemotaxis on angiogenesis and cancer cell migration.[5, 35] The versatile design allows for cell seeding arrangements in both 2D and 3D, application of shear stress or interstitial flow, and microscope access for continuous observation. In this study, a modified device, capable of imposing periodic uniaxial stretch without sacrificing the imaging capabilities, was developed to study the differentiation of embryonic stem cells (ESCs) into cardiomyocytes. With this platform, we were able to study how cyclic stretch affects the cardiogenesis process in a well-controlled microfluidic system.

Previous work has shown that murine cardiogenesis, involving the generation and manipulation of embryoid bodies (EBs), can be augmented both biochemically and biophysically. Biochemically, ascorbic acid, DMSO, retinoic acid, FGF and BMP2/4 are some of the growth factors that have been demonstrated to promote cardiogenesis.[1-3, 6, 14, 21, 23, 28, 36] Biophysically, control of EB size, electromagnetic stimulation and mechanical strain have also been shown to enhance cardiac differentiation.[9, 29, 30, 33, 34] In this study, we attempt to utilize a microfluidic system to impose biochemical and biophysical stimulations to EBs.

Microfluidic platforms have been shown to affect diffusion-dominated processes.[40] Yu et. al. demonstrated that cell proliferation rate was dependent on the height of the microchannels, presumably due to an accumulation of secreted factors. By comparing microchannels to conventional cell culture well plates, higher proliferation rates have been observed for murine

mammary gland cells and during murine embryo development.[24, 38] Since diffusion of growth factors have been shown to affect cardiogenesis, we hypothesized that cardiomyogenic differentiation will be enhanced in the confined space of microfluidic devices.

Another factor which influences cardiogenesis is mechanical stretch. Schmelter et. al. suggest that mechanical stretch activates the reactive oxygen species signaling pathway and thus enhances the differentiation of murine embryonic stem cells into cardiomyocytes.[30] Opposite results indicating that stretch inhibits differentiation have also been shown, attributed to the activation of TGF-β/Activin/Nodal pathway.[26] These conflicting results illustrate the need for further studies.

It is also important to note that current studies on EB cardiogenesis with stretch are limited to a two dimensional seeding condition. Three-dimensional environments, however, resemble more closely the native myocardial environment during development and myocardial infarct zones targeted for stem cell therapy.[12, 13, 16] Therefore, a microfluidic system which allows EBs to be seeded in 3D and experience cyclic uniaxial stretch might provide valuable new insights into the differentiation of embryonic stem cells into cardiomyocytes, and might also elucidate other important cellular behaviors where mechanotransduction is implicated.

Materials and Methods

Embryonic Stem Cell Culture and Differentiation

Murine embryonic stem cells (mESC) expressing a cardiac specific α-MHC promoter that was tagged with green fluorescent protein (GFP) (line CGR8, kindly provided by RT Lee, Harvard Medical School) allowed direct observation of differentiation into cardiomyocytes. To maintain ESCs in an undifferentiated state, Glasgow Minimum Essential Medium (GMEM) (Invitrogen),

supplemented with 1,000U/ml leukemia inhibitory factor (LIF, Sigma), 1mM Sodium Pyruvate (Invitrogen), 1x Non-Essential Amino Acid (Invitrogen), 15% Knockout Serum Replacement (Invitrogen), 25mM of HEPES, 10^{-4}M β-mercaptoethanol (Sigma), and 1x Penicillin-Streptomycin (Invitrogen) was used. Cells were maintained in flasks coated with 0.1% gelatin in PBS. Cell confluency was tightly controlled not to exceed 70%.

By removing LIF and creating a three-dimensional environment, mESCs spontaneously differentiated. The composition of the differentiation medium was identical to that of the maintaining medium except for the removal of LIF, the replacement of knockout serum by ESC Fetal Bovine Serum (Invitrogen), and the addition of 100μM of ascorbic acid.[36]

A standard hanging drop technique was used to induce differentiation.[27] Briefly, cell suspension solution was prepared at 10,000 cells/ml. 30μl drops were placed on the inside of a 100mm non-tissue culture treated Petri dish containing approximately 10ml of 1x PBS to prevent evaporation. Drops, containing small cell aggregates, were cultured for 2 days before being collected with a 10ml pipette. These aggregates were then cultured in differentiation medium for 3 more days for embryoid body (EB) formation.

For the experiments with BMP-2, 20μg/ml of BMP-2 antibody and 10ng/ml BMP-2 were supplemented into the medium on the first day of adherent culture in μFDs and well plates respectively. The concentration of BMP-2 was determined based on previous literature.[11] Both the antibody and BMP-2 were purchased from R&D Systems.

Human microvascular endothelial cells (hMVECs, Lonza) were cultured with complete EBM-2 (Lonza). Passages 4-7 were used for experiments with stretch stimulation.

Microfluidic Device Fabrication

μFDs comprised of three fluid channels separated by two gel regions were used to study differentiation. The gel regions allowed for three-dimensional seeding conditions. The design enables the application of uniaxial cyclic stretch without sacrificing existing advantages such as a well-controlled biochemical environment and good optical access. The thickness of the PDMS device in the gel region was 0.5-1mm, including thin PDMS films for microchannel enclosure. Finally, the rectangular shape allows for uniform strain application (Figure 1).

Embryoid Body Culture Conditions

For the 2D experiments, 5-8 EBs were seeded into μFD channels or onto 12-well plates coated with 0.1% gelatin. To seed EBs in 3D, stock collagen I solution derived from rat tail tendon (BD Biosciences) was mixed with 0.5N NaOH, 10x DMEM, water, medium containing 500 EBs/ml to produce a pH 7.4, 2mg/ml collagen I gel containing EBs. 10μl of gel were used for each microfluidic device.

Specific device preparation protocol and gel filling techniques have been described previously.[5] Briefly, μFDs were permanently bonded with plasma and coated with 0.1% gelatin. Then they were dried overnight in an 80°C oven to restore PDMS hydrophobicity. Channels were filled with differentiation medium after collagen gel had fully polymerized.

To study the effect of mechanical stretch, EBs were stretched for 24 hours at 10% strain and 1Hz 4 days after EB seeding in μFDs. EBs were subsequently cultured statically.

Motorized Stretch Apparatus Assembly

A precision linear motor (Parker MX80S, Irwin, PA) was selected to apply cyclic stretch to the μFDs based on required accuracy and precision of the travel range and travel velocity. To

prevent rust, the motor was enclosed in a stainless steel box. The μFDs were connected to the linear motor using a custom-designed clamp that could accommodate up to 4 μFDs for one set of experiments (Figure 2). One side of the clamp was firmly attached to the plates while the other side was connected to the linear motor.

Image Analysis, Quantification and Statistical Analysis

Both phase contrast and fluorescent images (20x) were taken with a Nikon Eclipse TE300 Microscope with Open Image™ Software. Individual embryoid bodies (EBs) were tracked and observed daily for GFP expression with identical exposure settings. The first day that GFP expression could be observed was defined as GFP1 and subsequent days as GFP2, GFP3 and so on. Images were taken daily and analyzed with Matlab. Without any contrast enhancement, a GFP-positive pixel was defined to be brighter than 120 on a 256 gray scale image. 120 was selected as the darkest level which could still be confidently identified as GFP positive. Most differentiation occurred on the flat parts of the adherent EBs that have spread outwards so the errors due to measuring the 2D projection were minimized. For the normalized data, each EB was individually normalized to its GFP expression on GFP1.

All data are presented as mean ± SEM. The Student's t-test was used to identify statistical significance ($p<0.05$). At least 20 EBs were examined and more than 3 independent samples were used for each condition.

Results

Validation of Motorized Microfluidic Platform

The compliant μFDs were connected to a precision linear stage which could be programmed to translate at specific frequencies and magnitudes (Figure 2). Cells seeded in the μFDs were stretched for 24 hours at 10% strain and 1Hz after they have fully adhered after 3 days.

Two different validation tests were initially conducted to ensure that cells actually experienced the imposed mechanical stimulation. First, we confirmed that the gel could withstand cyclic stretch without fracturing or detaching from the PDMS walls. A 2mg/ml collagen gel was injected into the μFD, allowed to polymerize, then subjected to cyclic stretch of different strains. At 12% strain, collagen gel in the device remained well adhered to the PDMS walls of the μFDs (Figure 3a). However, at larger strain (22%), the gel was clearly observed to detach from the walls. All subsequent tests with EBs were performed with a maximum of 10% cyclic strain.

A second test was designed to ensure that the cells were capable of responding to the mechanical stimulus. For this purpose, human microvascular endothelial cells (hMVECs) were cultured on the microfluidic channel surface and 10% strain, 1Hz uniaxial cyclic stretch was imposed (Figure 3b). Under these conditions, endothelial cells have been well documented to align perpendicular to the direction of strain.[18] Observed alignment was similar to what has been reported in literature, confirming that the μFD platform is capable of translating mechanical forces into cellular responses.

Effect of Culture Dimension

To examine the effects of confinement in the μFDs on cardiogenesis, compared with conventional culture plates, EBs were either seeded directly on the substrate – 2D – or embedded in a three-dimensional hydrogel – 3D (Figure 4a). GFP-positive and contracting cardiomyocytes were detected 48-72 hours after EBs were allowed to adhere to a substrate (Figure 4b).

Compared with well plates, the rate of increase in GFP was higher in both 2D and 3D seeding conditions (Figure 5). When EBs were cultured on 2D, the increase in GFP expression persisted over time in μFDs while those on well plates reached a plateau after three days. A higher rate of cardiomyogenic differentiation was also observed in μFDs as opposed to culture plates when EBs were suspended in 2mg/ml collagen I hydrogel.

The cardiomyogenic differentiation in μFDs was markedly diminished to a level similar to that in well-plates when bone morphogenetic protein 2 (BMP-2) was neutralized with a BMP-2 antibody (Figure 6a). Furthermore, the addition of BMP-2 in well plates enhanced cardiomyogenic differentiation (Figure 6b). BMP-2 was selected since blocking BMP-2 signaling has been shown to inhibit cardiac differentiation.[20, 39] This finding suggests that the secretion of BMP-2 may play a role in the enhanced cardiomyogenic differentiation in μFDs.

Effect of Mechanical Stretch

When exposed to 24hrs of uniaxial cyclic stretch at 10% strain and 1Hz, cardiogenesis of embryonic stem cells occurred significantly less as compared to the unstretched controls (Figure 7). The same phenomenon was observed for EBs cultured in 2D and in 3D. Moreover, EBs that did not express GFP prior to mechanical stretch failed to express GFP over time (data not shown). Note that the only difference between the static and stretched μFDs is the 24-hour uniaxial cyclic stretch on day 4 of adherent culture. μFDs were subsequently cultured statically in both cases. This suggests that even short term mechanical stretch interrupts the differentiation process and results in long term reduction of cardiogenesis.

Discussion

Embryonic stem cells have been considered as a cell therapeutic means to replenish myocardial infarction zones with functional cardiomyocytes.[8, 15, 37] It is, however, difficult to delineate the effect of the mechanical contraction of the heart on stem cell differentiation *in vivo* and many challenges remain in the differentiation, integration and incorporation of stem cells into the host tissue.[32] In this study, we have developed a compliant μFD to investigate the effects of culture dimension and mechanical stretch on the differentiation of murine embryonic stem cells into cardiomyocytes *in vitro*. This μFD includes three-dimensional hydrogel regions to provide a more realistic extracellular environment for cells *in vitro* and fluid channels to provide proper nutrient and waste transport, and gas exchange. It allows for the study of mechanical stretch, which has been implicated to be important in many biological processes, including the pulsation of the blood vessels from heart contractions to proper embryo development.[10, 17, 22] With an EB assay, this study aims to characterize the cardiomyogenic differentiation process in response to uniaxial cyclic stretch, with frequency and amplitude similar to the human heart.

We found that mechanical stretch at 1Hz and 10% strain on day 9 of differentiation yielded significantly less cardiomyogenic differentiation. The reduction in cardiomyogenic differentiation can be a result of direct inhibition of cardiomyocyte differentiation, reduction of cardiomyocyte proliferation, and/or alterations of differentiation rates. This finding confirms the results from two similar studies where the cardiomyogenic differentiation from ESCs was interrupted with cyclic mechanical strain.[25, 34] However, opposite findings have also been reported.[25, 26, 30] All the studies including ours illuminate the complexity of cardiomyogenic differentiation with mechanical stretch and the results can be highly dependent on the experimental setup. The strain magnitude, frequency, direction of strain, duration of stretch application, and at what stage of differentiation stretch is applied are all variables that need to be

systematically investigated. In our case, we chose the frequency and strain rates similar to what human cardiomyocytes experience *in vivo* intending to mimic the physiological environment.

Our findings suggest that mechanical stimulation disrupts the cardiomyogenic differentiation process prior to the expression of α-MHC, a late-stage marker for cardiogenesis and tagged with GFP in this study.[4] This is supported by two observations. First, GFP expression was never observed for GFP-negative EBs after stretch. Second, for EBs expressing GFP prior to stretch, the overall GFP expression did not increase after stretch, as it did in control. This can be due to the disruption of cardiomyogenic differentiation for cells yet to express α-MHC by the time of stretch stimulation. The combination of mechanical stimulation and fluorescent reporting system utilized here can further be used to elucidate the effect of stretch on the time course of cardiogenesis.

Another unique aspect of our study is the investigation of stem cell differentiation into cardiomyocytes in 3D. Most *in vitro* studies used commercially available systems involving a flexible membrane on the bottom of a well plate. Three-dimensional environments however resemble the *in vivo* conditions more closely. In our custom-built system, cells can be seeded in different conditions –on the μFD channels (2D) or suspended inside a collagen gel (3D). In the 2D scenario, cardiomyogenic differentiation was enhanced in μFDs, compared with that in well plates. Similar enhancement was observed when EBs were embedded inside collagen gel in 3D. The ability to study the influence of mechanical stretch in a highly controlled three dimensional microfluidic environment can be further used to study other biological processes.

In addition to the advantage of the versatility of cell seeding arrangement, μFDs inherently enhance the differentiation of embryonic stem cells into cardiomyocytes. This can be attributed

to an increased volumetric ratio. By estimating the amount of medium per EB (50µl for µFDs and 300µl for well plates,) growth factors are likely to be more concentrated in µFDs. Proliferation, demonstrated by immunofluorescent staining of ki-67, apoptosis, assessed with ethidium homodimer-1, and pluripotency, measured by Oct-4 immunofluorescence, are not significantly different between nonmicrofluidic and microfluidic conditions (not shown). This indicates that the enhancement of cardiomyogenic differentiation is not a result of an increase in cardiomyocyte proliferation, a reduction of apoptosis or changes in degrees of differentiation. The enhancement in µFDs suggests that µFDs may be a superior culturing platform than conventional well plates and the usage of a small culture dimension can be considered as an alternative to the addition of exogenous chemicals or adjustments of EB size to enhance cardiomyocyte differentiation.[3, 4, 7, 8]

Furthermore, we demonstrated that neutralizing BMP-2, a cell-secreted cardiogenic growth factor, reduced cardiomyogenic differentiation in µFDs and supplementing exogenous BMP-2 in well plates enhanced the cardiomyogenic differentiation. This confirms existing literature findings on the cardiogenic potential of BMP-2 in conventional well plates.[11, 14, 20, 31] This study illustrates the potential to utilize this microfluidic platform to investigate effects of growth factors in cardiomyogenic differentiation. In addition to BMP-2, there are many signaling molecules critical to the cardiomyogenic process and their cardiogenic effects can also be examined with this microfluidic platform.

This study deploys a well-controlled three-dimensional microfluidic system to study the cardiogenesis process of murine ESCs with an EB assay. The effect of culture dimension and uniaxial strain were characterized. First, we demonstrated that a higher EB to media ratio in µFDs led to enhanced cardiomyogenic differentiation and the inhibition of BMP-2 with a

neutralizing antibody reduced the enhanced cardiomyogenic differentiation in μFDs. Furthermore, uniaxial cyclic stretch at 10% strain and 1Hz on day 9 was found to have a negative impact on cardiomyogenic differentiation. These findings provided additional insights on biophysical factors with which cardiogenesis could be affected.

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Figure Legends

Figure 1: Design of a compliant microfluidic device capable of withstanding cyclic stretch while maintaining optical access: (a) schematic illustration of the μFD; (b) top view of the μFD; (c) side view of the clamp and the microfluidic devices

Figure 2: Design of the stretch platform: (a) schematic diagram illustrates that the stretch apparatus was placed in the incubator; (b) displacement could be precisely controlled

Figure 3: Three validations of proper functions of the motorized microfluidic system. (a) assessment of hydrogel detachment with cyclic stretch and gel remained adhered to the wall at 12% strain but detached at 22%; (b) hMVECs aligned perpendicular to 10%, 1Hz strain, as reported in the literature. Scale bar: 100μm

Figure 4: Procedure of differentiation and representative images of differentiated cardiomyocytes: (a) procedures of ESC differentiations, EB formations and seeding conditions (b) representative images of GFP-positive areas of EBs over time. Scale bar: 200μm

Figure 5: ESCs exhibited enhanced cardiomyogenic differentiation in μFDs: higher differentiation was observed in μFDs compared with conventional well plates, both in 2D (a) and 3D (b). Asterisks and diamonds (◊) represent statistical significance ($p<0.05$) of the absolute and normalized GFP expressions between the two conditions respectively.

Figure 6: Effect of BMP-2 on cardiomyogenic differentiation: (a) BMP-2 was neutralized with the addition of a neutralizing antibody (20μg/ml) in μFDs; (b) exogenous BMP-2 (10ng/ml) was added in the well plates. Asterisks and diamonds (◊) represent statistical significance ($p<0.05$) of the absolute and normalized GFP expressions between the two conditions respectively.

Figure 7: Uniaxial cyclic stretch inhibited ESC differentiation: in both 2D (a) and 3D (b), ESCs had inhibited differentiation. Asterisks and diamonds (◊) represent statistical significance ($p<0.05$) of the absolute and normalized GFP expressions between the two conditions respectively.

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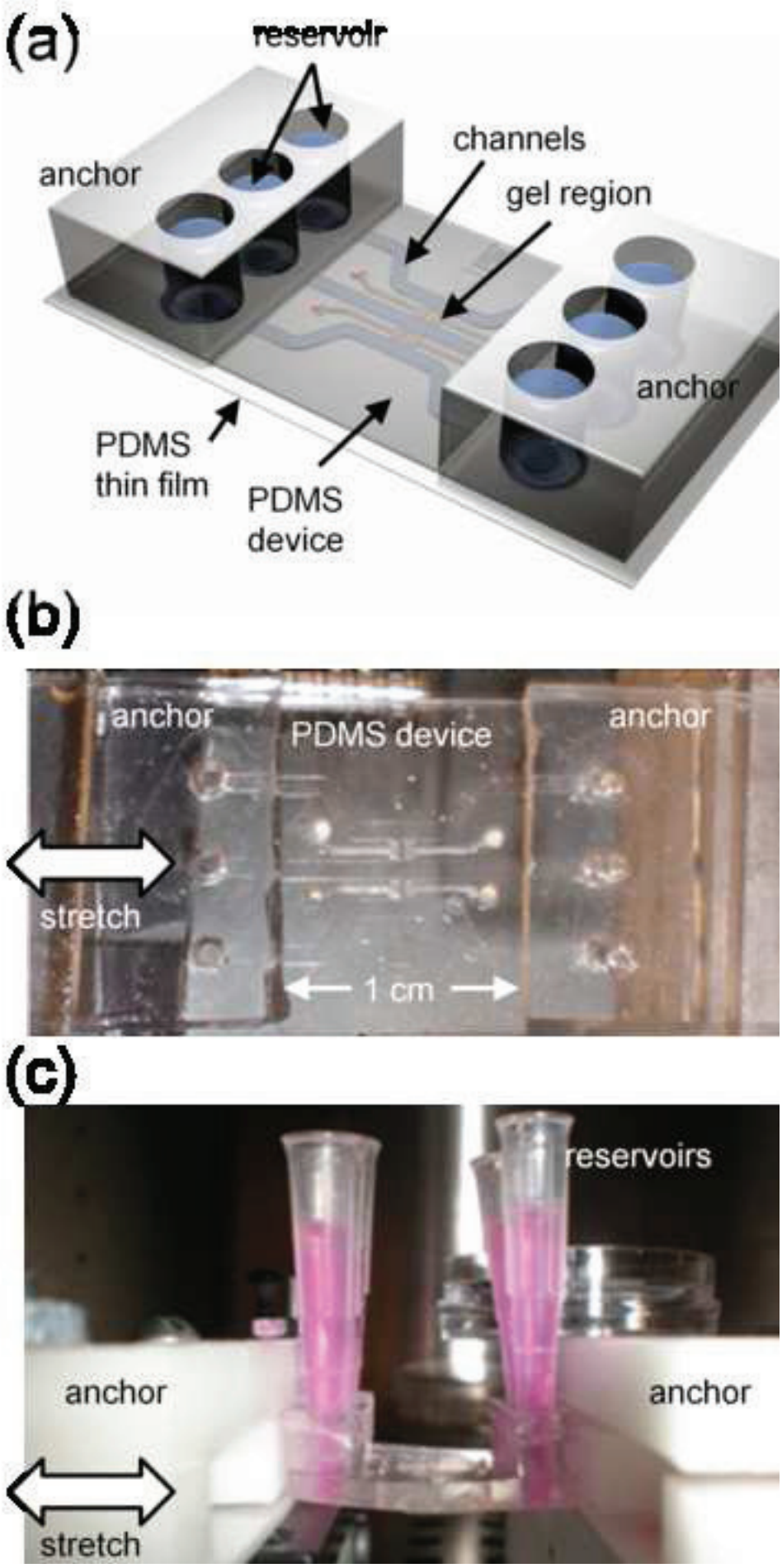

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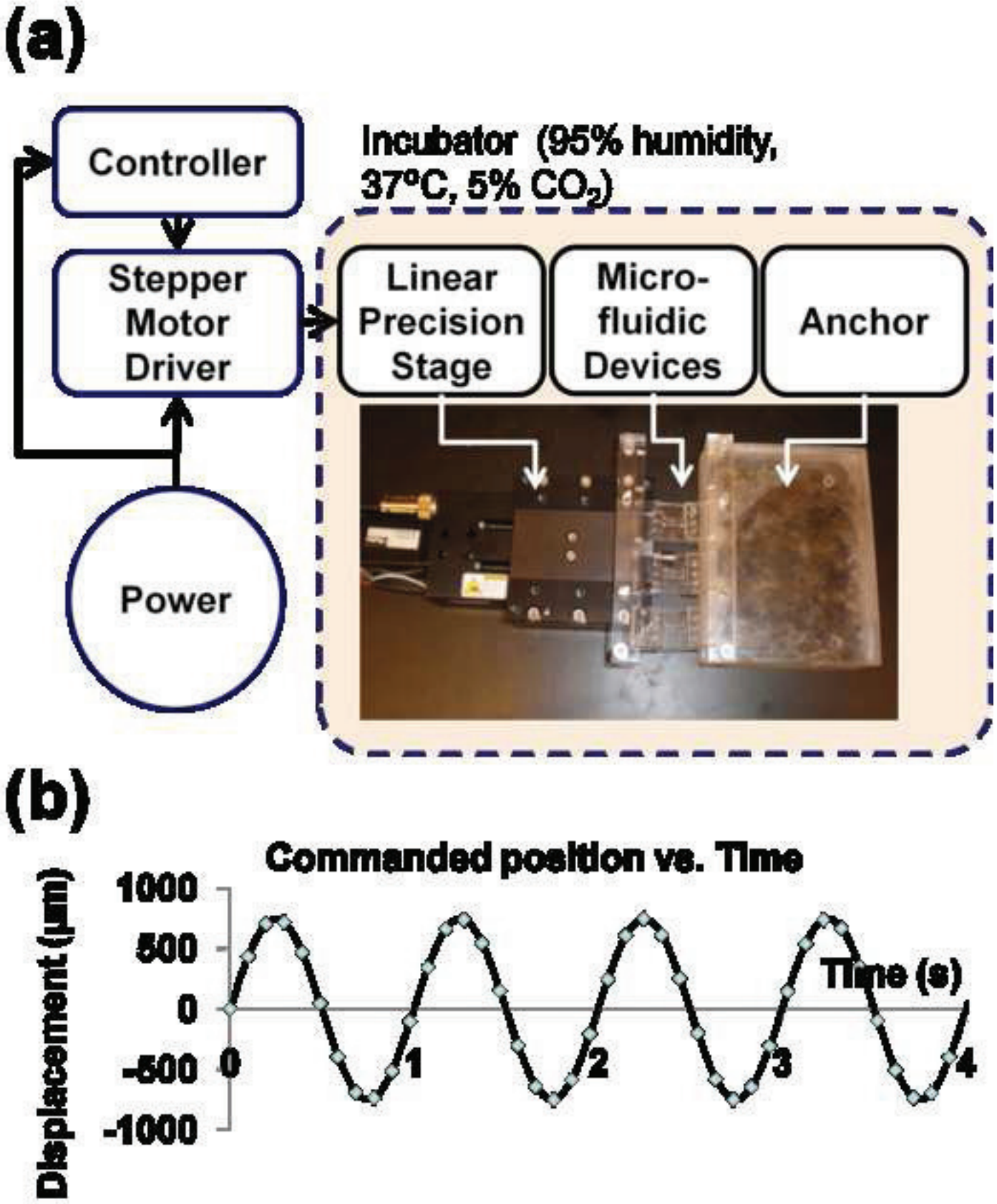

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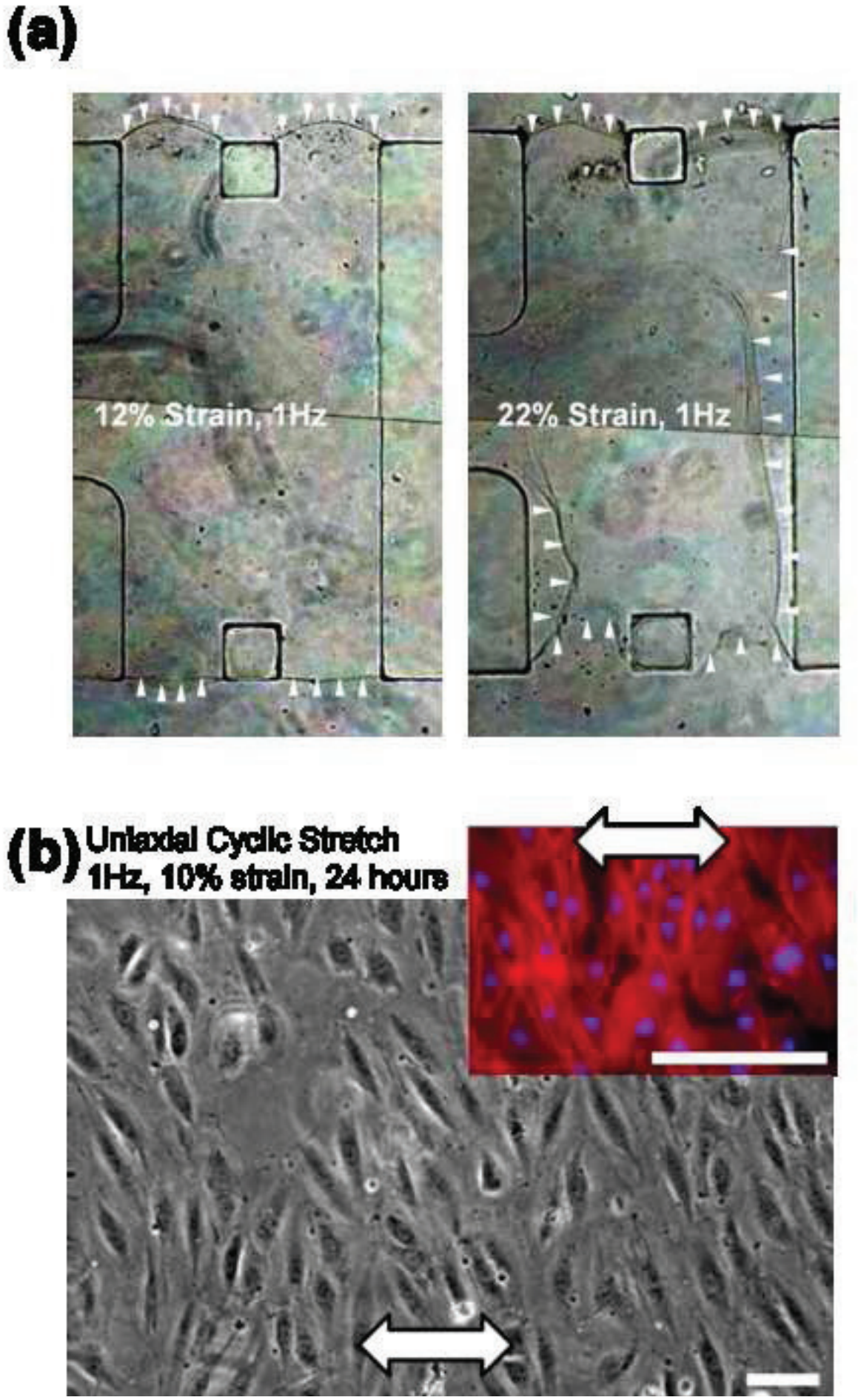

Figure 4

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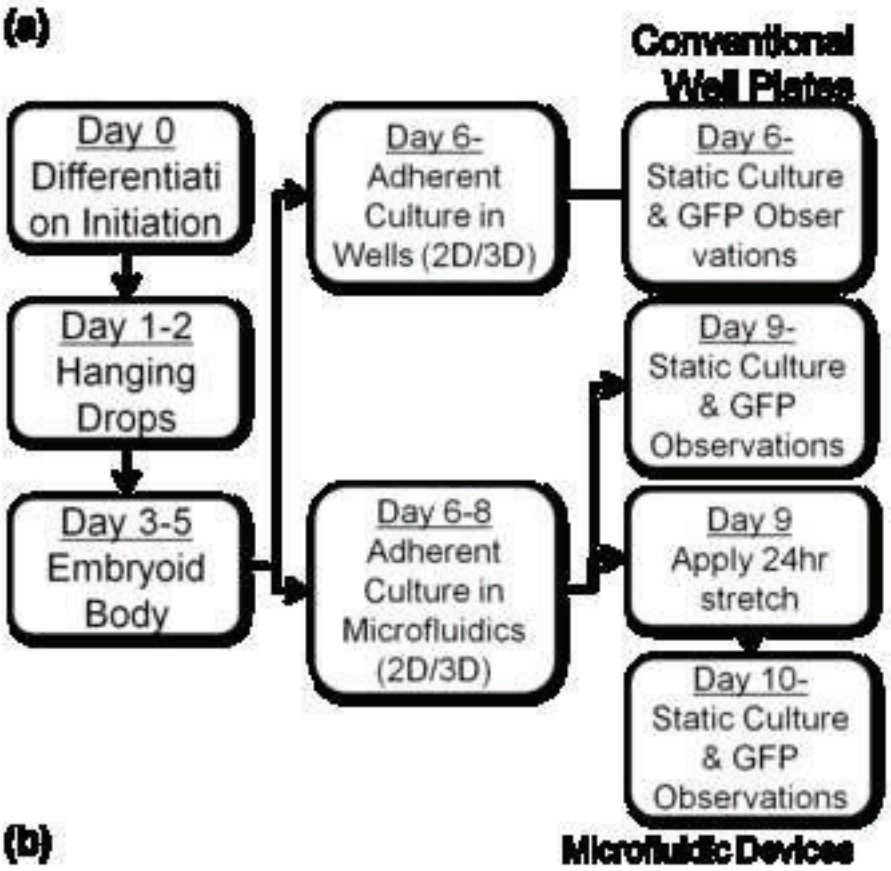

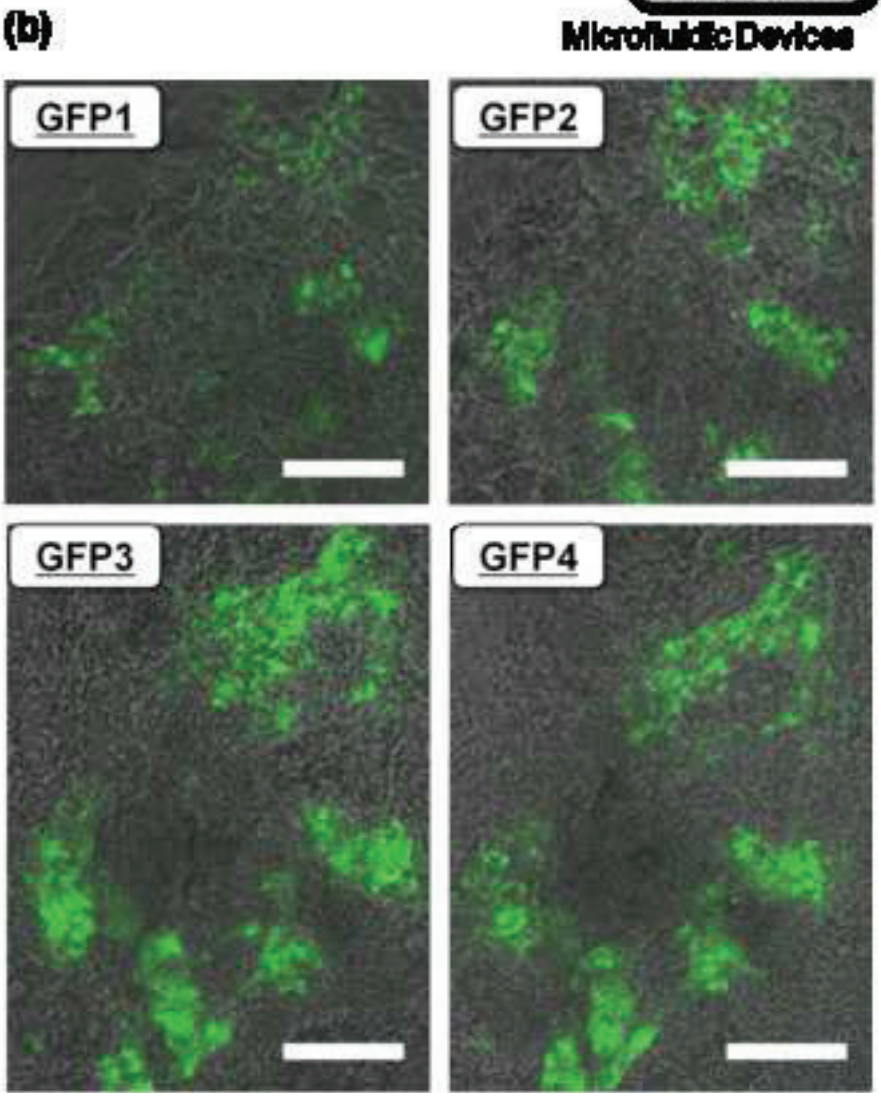

Figure 5

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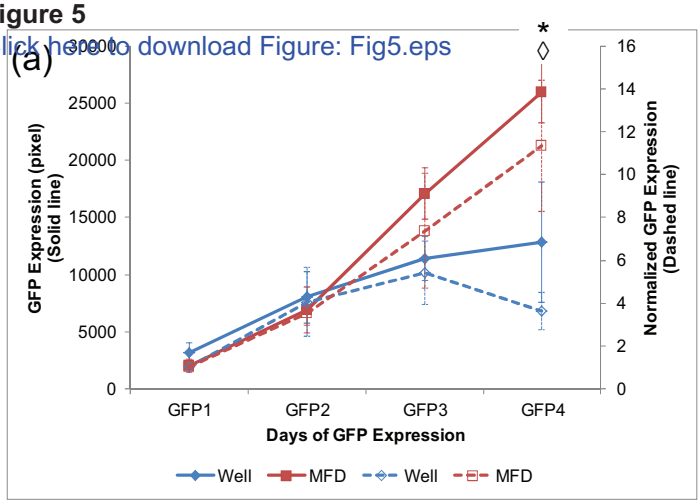

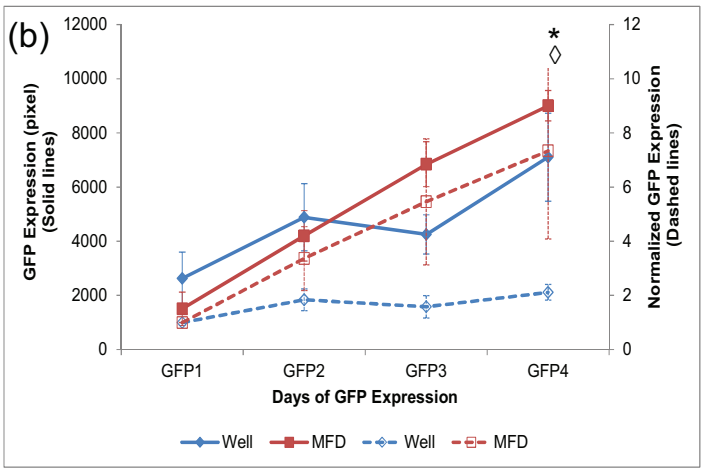

Figure 6

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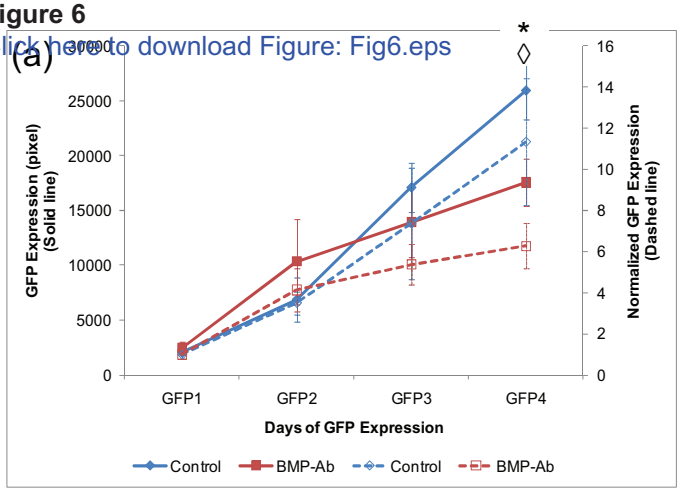

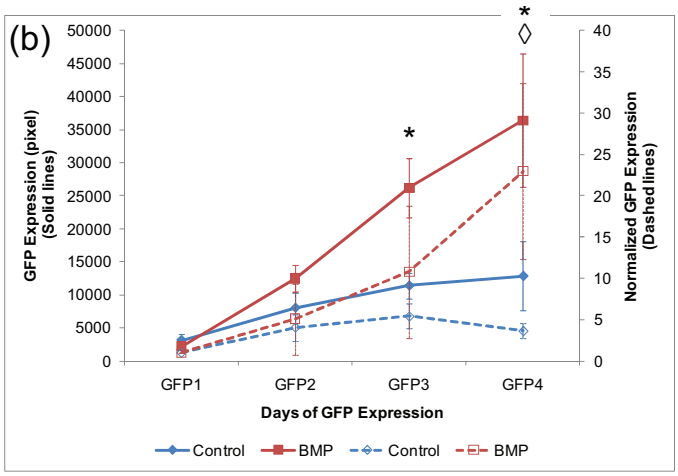

Figure 7

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