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Large particle multiphoton flow cytometry to purify intact embryoid bodies exhibiting enhanced potential for cardiomyocyte differentiation

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Abstract

Embryoid Bodies (EBs) are large (> 100 μm) 3D microtissues composed of stem cells, differentiating cells and extracellular matrix (ECM) proteins that roughly recapitulate early embryonic development. EBs are widely used as *in vitro* model systems to study stem cell differentiation and the complex physical and chemical interactions contributing to tissue development. Though much has been learned about differentiation from EBs, the practical and technical difficulties of effectively probing and properly analyzing these 3D microtissues has limited their utility and further application. We describe advancement of a technology platform developed in our laboratory, multiphoton flow cytometry (MPFC), to detect and sort large numbers of intact EBs based on size and fluorescent reporters. Real-time and simultaneous measurement of size and fluorescence intensity are now possible, through the implementation of image processing algorithms in the MPFC software. We applied this platform to purify populations of EBs generated from murine induced pluripotent stem (iPS) cells exhibiting enhanced potential for cardiomyocyte differentiation either as a consequence of size or expression of NKX2-5, a homeodomain protein indicative of precardiac cells. Large EBs (330–400 μm , diameter) purified soon after EB formation showed significantly higher potential to form cardiomyocytes at later time points than medium or small EBs. In addition, EBs expressing NKX2-5 soon after EB formation were more likely to form beating areas, indicative of cardiomyocyte differentiation, at later time points. Collectively, these studies highlight the ability of the MPFC to purify EBs and similar microtissues based on preferred features exhibited at the time of sorting or on features indicative of future characteristics or functional capacity.

Keywords

Embryoid body; cell aggregate; stem cells; cardiomyocytes; flow cytometry

Introduction

Pluripotent stem cells, isolated from mammalian embryos or induced from mature cell types, are highly proliferative and can differentiate into most cell types of the body¹⁻³. These attributes render them attractive for studying tissue development or the pathogenesis of disease progression as well as for drug testing and cellular therapies. Effective control of stem cell state largely dictates the utility of pluripotent stem cells for research and clinical application. Thus, significant research effort has been dedicated to delineating mechanisms that either maintain pluripotency⁴ or drive differentiation⁵. The embryoid body (EB), an aggregate of pluripotent stem cells that roughly recapitulates the complex assembly of cell-cell and cell-matrix adhesions and corresponding intercellular signaling of embryogenesis, is commonly utilized to drive differentiation *in vitro*. Although more defined methods have been developed to direct differentiation of individual cell types, such as two-dimensional formats (i.e., monolayer culture) with defined soluble (e.g., growth factors, recombinant DNA encoding essential transcription factors) and/or defined insoluble substrates (e.g., extracellular matrix proteins), the EB remains an invaluable *in vitro* model to study the complex signaling interplay that impacts the state of a stem cell or stem cell progeny with 1) development, 2) extended *ex vivo* culture or 3) following transplantation to a native tissue.

The primary challenge of studying cells of the EB is their propensity to change state after common research manipulations such as the addition of exogenous labels (e.g., antibody staining)⁶ and disaggregation⁷⁻⁸. Therefore, to probe interior cells of the EB, investigators were previously limited to terminal, low throughput cell and molecular biology techniques including staining of histologic sections and PCR analysis of extracted RNA. More recently, the development of fluorescent reporter constructs coupled to differentiation-specific gene expression and combined with advanced imaging modalities capable of non-invasively imaging deep within a biological specimen (e.g., confocal and multiphoton fluorescence microscopy), enables investigators to probe cells in the interior of living EBs⁹⁻¹¹. To enhance the throughput of this type of analysis, we recently coupled multiphoton laser scanning microscopy¹² with a microfluidic-based flow cell, creating an imaging flow cytometry system (Multiphoton Flow Cytometer, MPFC)¹³ capable of non-invasively detecting size and fluorescence properties deep in the interior of large intact multicell aggregates, such as EBs. This was an important advance as large numbers of EBs can be quickly assessed, improving study power as well as the ability to distinguish important biological events from noise without relying on expensive arrays or time-consuming serial imaging.

Analysis of EBs would be further improved if the EBs could be purified based on characteristics that precede and/or predict a differentiation state of interest without disrupting EB organization. One relevant application pertains to the differentiation of cardiac cell types for the recovery of lost myocardial function. This application dictates a need for identifying and examining cellular aggregates with enhanced cardiac potential to understand the environment, signals and corresponding mechanisms that contribute to cardiac differentiation. For example, it has been shown that EB size can influence the proportion of cells that enter the cardiac lineage¹⁴⁻¹⁵, thus identifying and segregating EBs on size soon after EB formation could provide a population of EBs with increased cardiac potential. Similarly, the presence of cardiac progenitor pools has been linked to the generation of mature cardiac cell types and not unwanted cell types *in vivo*¹⁶. Unknown, however, are the conditions that contribute to behaviors of these cardiac precursors, including proliferation, migration and differentiation into individual cardiac cell types. Thus, the ability to purify EBs based on characteristics early in development (i.e., size or presence of cardiac precursors) associated with generation of a particular cell type while maintaining

aggregate viability and structure would provide an important tool for subsequent studies of differentiation.

Here we demonstrate the ability to purify intact murine iPS-derived EBs based on size or intensity of a fluorescent reporter corresponding to expression of NKX2-5, an early cardiac transcription factor¹⁶. Purified EBs were monitored over several days to determine whether developmental outcome, namely the generation of mature cardiomyocytes, corresponded to size or reporter expression. To enable this study, we enhanced the detection software of our previously described MPFC system¹⁷ to permit sorting of EBs based on real-time measurements of size and spatially segregated fluorescence. As a result, we show that purified populations of EBs with high NKX2-5 expression at early time points in differentiation are more prone to form beating areas at later time points. Furthermore, the largest EBs within a broad size distribution have the highest potential for forming beating areas and producing the highest percentage of cardiomyocytes per EB.

Results

Utilization of a segmentation algorithm for real-time measurement of non-contiguous fluorescence intensity

Previously, we used the MPFC to sort EBs based on contiguous fluorescence intensity within an EB¹⁷ so that EBs could be effectively distinguished from fluorescent noise. This was accomplished by coupling our in house developed laser scanning acquisition software, WiscScan, with ImageJ-based¹⁸ analysis tools for real time large particle analysis in flow. ImageJ was chosen due to its wide array of existing analysis routines, ease of adaptability and customization and ability to run both within WiscScan at acquisition or as a standalone application for further analysis after acquisition. The ImageJ analysis routine, “Analyze Particles” was utilized to measure connected clusters of pixels above background intensity. In this way, the size and intensity of large particles with continuous fluorescence throughout the particle could be determined. However, this implementation could not define the border of a large particle in real-time in the absence of fluorescence intensity. This was disadvantageous as it limited the utility of MPFC as different collection speeds, variable fluorescence expression levels or changes in focus can all affect detectable fluorescence at the periphery. To overcome this limitation a beneficial optical feature of the MPFC was exploited namely, the ability to acquire fluorescence data through multiphoton excitation while simultaneously collecting a bright field image through the use of a transmitted light photodetector. The bright field data was used to distinguish EB borders and thereby EB size without relying on the presence of fluorescence signal. To do this, the MPFC data collection software was modified to again take advantage of pre-existing ImageJ functions to reliably define the size and spatial location of an EB within the bright field image (Figure 1A). To begin, the “Find Edges” function was used to highlight the border of the EB in the bright field image, creating an outline of the particle. To ensure that small edge scan effects did not significantly affect the robust measurement of the EB or particle border, the “Gaussian Blur” function was used to smooth the highlighted borders in the image. The sigma radius input for the Gaussian blur defines the degree of smoothing. For EBs, a sigma radius of 2.2 was effective for accurate estimation of particle size. A higher sigma radius resulted in a more drastic overestimation of particle size, while a lower sigma radius led to more frequent instances of discontinuities in the border, resulting in inaccurate measurements. From the resulting outline of the particle, the minimum autothreshold feature was employed, creating a mask of all pixels within the particle. This mask was approximately 10% larger than the actual area of the particle (data not shown). To correct for this overestimation an erode function was applied that strips the border pixels of the mask and minimized error to $4.0\% \pm 4.6\%$ of the total area, as determined by manual tracing. This measurement was displayed in a results table generated in real-time as area in pixels, which could be displayed as area in

microns based on MPFC system calibration for the chosen optical parameters (i.e. lens magnification, zoom, etc.).

The above-described analysis of the bright field data not only allows for effective robust sorting of particles, but can directly assist in the analysis of fluorescence-based trends in the corresponding fluorescence intensity image. The MPFC software not only can simultaneously collect the bright field and fluorescence intensity images but can also conduct co-registered analysis on both so that the bright field-based morphology detection can be used to directly assist with fluorescence data interpretation. Masking the border of an EB in flow in the bright field image allows for a measurement of particle size in real time and also creates a region of interest (ROI) that can be applied to the fluorescence image to confine and sum intensity pixels that are not contiguous, thereby permitting measurement of the fluorescence intensity per unit area. To calculate fluorescence intensity per unit area (Figure 1B), the fluorescence intensity image acquired through WiscScan and then regressed with a background threshold so that only pixels above background intensity appear on a processed intensity image. Due to additional noise produced by flowing media and cell debris, there may be fluorescent pixels outside of the particle that exceed the background threshold. To exclude these confounding pixels, the ROI created from segmentation of the bright field image was applied to the processed intensity image, resulting in an image displaying only the pixels above background intensity that were inside the particle. Each pixel within the ROI was assigned an intensity value between the background threshold and maximum detectable intensity value (255). The sum of all the pixel intensity values provided a measurement of total intensity, which was then divided by the ROI for a final measure of fluorescence intensity per unit area. Similar to the size measurement, fluorescence intensity could be displayed as both area in pixels or microns. All code for the MPFC sorting is open source (<http://loci.wisc.edu/software/wiscscan-flow-cytometry>).

Purification of EBs based on size

To determine whether the bright field segmentation algorithm coupled to the MPFC could effectively detect and sort EBs based on calculated size, EBs were sorted into three different size ranges (Figure 2A). To this end, hanging drops were seeded at 200, 400, and 500 cells per drop, to obtain a relatively wide size distribution of EBs. EBs of different cell number were combined into a single population and the size distribution determined in a pre-sort analysis trial (Figure 2B). Consecutive sorting of a given population of EBs was performed to obtain small, medium and large EB fractions (Figure 2C). First, a threshold of 330 μm was set, separating EBs greater than 330 μm into the sorting outlet port and allowing EBs less than 330 μm to flow into the main outlet port. EBs collected in the main outlet port were further sorted such that EBs greater than 250 μm (and necessarily less than 330 μm) were diverted to the sorting outlet port. Sorting efficiency was defined as the number of EBs physically collected from the sorting port relative to the number of positive events detected in flow by the software. The enrichment ratio was defined as the ratio of the number of desired EBs to undesired EBs in the sorting output port divided by the same ratio at the sample input port¹⁹. Sorting efficiency of EBs greater than 330 μm in diameter (large) and of EBs between 250 and 330 μm (medium) was $94.1 \pm 5.6\%$ and $87.9 \pm 19\%$, respectively while enrichment ratios were 18.0 ± 15.7 and 25.0 ± 22.5 , respectively (Figure 2D). Therefore, real-time segmentation of the bright field image of an EB yields an accurate measure of size as well as subsequent sorting capabilities based on that size determination.

Sorting EBs based on NKX-2.5 GFP reporter expression indicative of cardiac precursor cells

To test the ability of the MPFC to purify EBs based on varying levels of non-contiguous fluorescence intensity, we generated EBs from mouse induced pluripotent stem cells (iPSCs)

carrying a NKX2-5 Emerald Green Fluorescent Protein (EmGFP) Bacterial Artificial Chromosome (BAC) reporter^{16, 20}. EBs matured for 3 days in hanging drops and then were sorted based on EmGFP fluorescence intensity with the MPFC (Figure 3A). To establish a threshold such that EBs with the greatest EmGFP intensity per unit EB area (i.e., the top 25%) would be positively sorted, a histogram depicting fluorescence intensity per unit area of a test sample population of EBs was generated under analysis flow conditions (Figure 3B). During sorting flow conditions, EBs below the determined threshold were directed to the main outlet port while those above the threshold were directed to the sorting port (Figure 3C). After sorting, EBs from both the main and sorting outlet ports were collected and counted before transferring to 6-well plates for ensuing culture. Sorting efficiency was defined as the ratio of the number of EBs physically collected from the sorting port, to the number of positive events detected in flow. A sorting efficiency of $89.0 \pm 11.1\%$ and an enrichment ratio of 25.4 ± 19.3 was achieved (Figure 3D), which are comparable to previously reported values corresponding to the purification of large fluorescent beads or EBs uniformly stained with CellTracker™ (Molecular Probes/Invitrogen, Carlsbad CA) dye¹⁷.

EBs sorted at early timepoints according to size or NKX2-5 expression show a higher propensity to develop mature cardiomyocytes

EB size can dictate yield of differentiated cardiomyocytes²¹⁻²³ demonstrated by studies attempting to control initial EB size with cell culture constraints. Another approach to control size of microtissue populations is to sort them after formation using mechanical barriers to microfluidic channels of prescribed dimension²⁴. The primary limitation of these methodologies is the need to adjust physical dimensions of the culture vessels or sorting device to obtain a final population of EBs within specific size ranges tailored to a user-specific application. Here we have shown that the modified MPFC is capable of sorting EBs in the range of 100 - 400 μm with high efficiency. To determine whether the size of EBs sorted in our system impact their ability to produce cardiomyocytes, we isolated three distinct size ranges (small, 250 μm in diameter or less; medium, 250-330 μm ; large, 330 μm or more) using the MPFC system (Figure 2). The mean diameters of the resulting sorted populations were $209 \pm 50 \mu\text{m}$, $298 \pm 34 \mu\text{m}$, and $347 \pm 29 \mu\text{m}$, which all significantly differed from one another ($n > 50$ for each of 3 replicates, $P < 0.05$) (Figure 4A). The large size group of EBs produced the most beating areas on day 7 of differentiation (1.63 ± 0.36 , Figure 4B), and significantly exceeded the number of beating areas produced by the medium (0.52 ± 0.36 ; $P < 0.05$) or small fractions (0%, $P < 0.05$). Similar trends were found when quantifying cTnT, a protein marker expressed only in mature cardiomyocytes, with the large EB group (1.00 ± 0.24) having significantly greater cTnT expression per EB compared to the medium (0.25 ± 0.22 ; $P < 0.05$) and small fractions (0; $P < 0.05$) (Figure 4B). Thus, larger EBs separated on the MPFC at an early time point in differentiation exhibit enhanced potential for cardiomyocyte differentiation.

We also hypothesized that EBs expressing relatively high levels of the early cardiac transcription factor NKX2-5 at early time points in differentiation would produce significantly higher yields of mature cardiomyocytes at later time points in differentiation. As before, day 3 EBs were introduced into the MPFC and those with the top quartile of GFP fluorescence intensity were sorted. The relative amount of NKX2-5 expression in the sorted population compared to the unsorted populations was 1.25 ± 0.01 a.i.u. and 0.75 ± 0.17 a.i.u., respectively (Figure 4C) ($n > 50$ for each of 3 replicates, $P < 0.05$). EBs were plated after sorting and at day 7 after EB generation (4 days after sorting) were assessed for beating areas and cTnT expression. Sorted EBs produced significantly more beating areas compared to the unsorted population of EBs (1.31 ± 0.12 and 0.86 ± 0.20 ($P < 0.05$), respectively, Figure 4D). Sorted EBs had higher expression levels of cTnT compared to the unsorted

population (1.00 ± 0.34 and 0.85 ± 0.17 a.i.u., respectively), though these values were not significantly different. Thus, sorting EBs with our enhanced MPFC system at an early time point in EB development based on cardiac precursor gene expression can be used to predictively enrich for EBs with a higher propensity to form beating areas indicative of cardiac differentiation.

The MPFC is also capable of two-parameter sorting and so we tested whether EBs could be further enriched for cardiomyocyte differentiation by simultaneously isolating larger ($> 270 \mu\text{m}$ diameter) EBs with high levels of NKX2-5 (Figure 4E). Once again, sorted EB populations produced significantly more beating areas compared to unsorted EB populations (1.30 ± 0.32 and 0.87 ± 0.20 a.i.u., $n > 50$ for 3 replicates, respectively, Figure 4F). Sorted EBs had higher expression levels of cTnT compared to those of the unsorted population (1.00 ± 0.25 and 0.70 ± 0.16 a.i.u., respectively, Figure 4F), though these values were not significantly different. In this case, sorting based on two characteristics of EBs did not further enrich for cardiomyocyte differentiation. In fact, the enrichment was not statistically different than either enrichment based on size or based on NKX2-5 expression ($P = 0.2$ and $P = 0.5$, respectively). These data suggest that either 1) the populations overlap or 2) that the NKX2-5 positive EBs comprise a subset of the “large” EB population and that the enrichment based on size is partially diminished by the EBs that give rise to other cardiac cell types (i.e., NKX2-5 progeny) but not cardiomyocytes.

Discussion

Here, we take advantage of real-time image processing tools incorporated into the MPFC to sort EBs based on size as well as non-contiguous fluorescence intensity, corresponding to expression of cardiac-related transcription factor, NKX2-5, and preserve sorted populations for long term analysis of differentiated cell function. This is the first reported application of real-time image segmentation to trigger sorting in a microfluidic device for large ($> 100 \mu\text{m}$) cellular particles. Although the images in this study were acquired using a multiphoton laser scanning microscopy system, the size-sorting approach could be utilized with any imaging modality capable of producing a bright field image of large particles or capable of simultaneously collecting bright field and fluorescence images (e.g., confocal microscopy²⁵). Our motivation for developing the MPFC system was driven by a desire to understand the factors that stimulate cardiomyocyte differentiation. However, the approach could be tailored to study many cell types and associated behaviors in aggregates or microtissues. Thus, we describe an accessible and versatile system that can help transform the way that adherent cell types are studied in the context of three-dimensional microenvironments.

EB size is known to influence the type and amount of differentiated cells present in the EB over time. For example, Mohr et al¹⁴, show that smaller EBs (formed in $100 \mu\text{m}$ microwells) are less likely to form beating cardiomyocytes than those formed in larger microwells ($300 - 500 \mu\text{m}$), but that those smaller EBs with contracting areas are relatively enriched in cardiomyocytes compared to larger counterparts. Similarly, others found that cardiogenesis and neurogenesis were regulated by the size of the concave microwell used to generate EBs^{15, 22}. These studies were accomplished by regulating size at the time of EB formation; less well studied are phenotypes emerging when EBs are sorted at points following EB formation. A microfluidic device was recently described²⁴ which utilized a series of appropriately spaced pillars in a microchannel to alter the fluid flow path to divert and thereby purify EBs with diameter differences of approximately $100 \mu\text{m}$, with a maximum size of approximately $300 \mu\text{m}$. This device is inexpensive and accessible to many labs but is limited by the physical dimensions of the device such that multiple devices would be needed depending on the cell aggregate size and range of interest. Our MPFC system can

handle a large range of EB sizes, the upper limit of which is dictated by the size of the channel. For the device described here, the height is 1000 μm and represents the smallest dimension of the flow cell cross-section. Thus, this device can accommodate EBs of approximately 50 μm up to 500 μm in diameter (approximately half the height of the flow cells¹⁷). In addition, the MPFC can discriminate differences as low as the error corresponding to real-time size measurement (4.0% + 4.6% of the manually measured diameter; e.g. 26 μm for a 300 μm particle). We show that differences in EB size at three days after EB formation significantly alter the percentage of beating areas in a given EB population and the corresponding expression of cardiac troponin T. Studies of this type could be used to determine how factors related to size, such as diffusion of soluble molecules (e.g., growth factors or metabolites) and insoluble structural elements, such as extracellular matrix proteins, impact cardiomyocyte differentiation.

Reporter elements coupled to proteins indicative of stem cell differentiation enable identification of soluble and insoluble factors that direct differentiation. The utility of these reporter cell lines is augmented when stem cells and their progeny can be maintained in a 3D or tissue-like arrangement, as that found in an EB or similar microtissue. One could argue, however, that much progress has already been made in understanding factors that direct stem cell differentiation. Indeed, recent progress indicates gene delivery of transcription factors can directly program murine stem cells to a cardiomyocyte fate with high efficiency¹⁶. By comparison, the MPFC technology described here may not provide an effective means to optimize the yield of cardiomyocytes. It can, however, provide key insights into stem cell microenvironments (i.e. compositions of EBs) that are conducive to differentiation and that maintain a differentiated and functional phenotype. In this way, we can continue to improve extended *in vitro* culture conditions and perhaps better predict and promote outcomes of cell delivery *in vivo*.

The MPFC technology described here has advantages for sorting EBs based on size or reporter elements over previously described systems^{24, 26}, including our first generation MPFC device^{13, 17}. Our first generation device was accessible to laboratories with access to microfabrication capabilities and imaging systems with real time data acquisition capabilities. It was unique in that it allowed for sheathless particle focusing with minimal damage to the large particle as a consequence of flow and could sort intact EBs based on intrinsic or extrinsic fluorescence signals. The current generation MPFC described here maintains these advantages and adds the ability to analyze non-contiguous fluorescence of an EB in real time and sort based on those fluorescence parameters. The sorting efficiency is comparable to the first generation sorting device, with average efficiency of 85-95%. Future iterations of the MPFC will seek to take advantage of the images acquired of each EB in flow. Because an image is obtained, it will be possible to utilize ImageJ functionality to adapt image processing approaches to sort based on distribution of fluorescence, and so associated protein or molecule, within an EB (or other large particle).

Sorting efficiency of the first generation MPFC as well as the current generation is limited by false positive sorting events, which most often reflect coincidence events when two or more particles are detected in the same image frame. In our device, coincidence occurs in approximately 7% of events detected by the software (data not shown). Coincidence increases with particle-particle interactions. We attempt to limit particle-particle interactions by separating them with inertial lift forces imposed by the asymmetric curving channels²⁷ present in the microfluidic device. However, coincidence does still occur and changes as a consequence of differences in particle velocity. Additional factors that may increase the false positive rate may include the unpredictability of the velocity of smaller EBs in microfluidic devices. Velocity variability can also result in false negative events if EBs are diverted outside the interrogation region and therefore never trigger a sorting pulse. We have

empirically verified that as particle size decreases the corresponding velocity range broadens. Most of the sorting errors evident in our system could be reduced or alleviated if particle velocity was increased because the increase in velocity would improve particle focusing and separation within the microfluidic device²⁸. Currently, we are limited by a relatively low maximum image acquisition speed (~5.5 frames/sec) and corresponding low resolution (128×128) of our optical arrangement and so flow velocities cannot exceed approximately 2 mm/s. To this end, we are investigating line scanning and other high-speed software and hardware scanning approaches to improve imaging acquisition rates and thereby improve both throughput and sorting efficiency.

Another consideration when analyzing spatially segregated fluorescence intensity within a microtissue on the MPFC, is the variability corresponding to each detected optical section. As a microtissue traverses the interrogation region, multiple planes will be measured based on the rotational position of the EB at the instant of an image scan. To determine the approximate variability of NKX2-5::GFP expression in our EBs, we obtained images of optical sections at random 3D orientations of single EBs in static mode, in an attempt to simulate the various possible optical planes detected in flow. We found that the coefficient of variation for a measurement of NKX2-5 fluorescence of a single EB is approximately 25 + 13% (n=13). As expression levels of NKX2-5 increase, there is more variability in the measurement (Supplementary Figure 1), which is not surprising considering “pockets” of differentiating cells are typically present in EBs, and not uniformly distributed throughout the volume. Here, the impact of sorting based on one z-plane measurement did not drastically affect the outcome of this specific population of EBs and associated reporter element, but could have a greater affect on other cell types and associated reporter elements if expression of the reporter is localized to one side of the EB, or expressed as a gradient from one side to another. Future MPFC software improvement will include the ability to average measurements from multiple z-planes of an EB or other large particle. At current flow rates EBs are most commonly detected in three consecutive frames as they traverse the interrogation region. Averaging intensity measurements from these three frames would provide a more representative quantification of the total fluorescence intensity throughout the volume of the large particle.

Conclusion

Our results demonstrate that MPFC technology can be used to effectively sort EBs based on fluorescence of cells within the EB, even if fluorescent cells are separated from one another. The technology can help advance our understanding of the factors that impact on behavior of cells within the EB which should translate to a better understanding of what happens to differentiated or differentiating stem cells after transplantation to a tissue bed or with extended culture ex vivo. In addition, the MPFC could be used to augment the study of stem cell behavior in other engineered 3D constructs or the behavior of other cell types in aggregate or microtissue formats by increasing throughput and utilizing unique features of multiphoton excitation microscopy to probe endogenous fluorescent structures including collagen and autofluorescent metabolites.

Experimental

Cell Culture

Murine iPS cells carrying a NKX2-5 Emerald Green Fluorescent Protein (EmGFP) Bacterial Artificial Chromosome (BAC reporter), referred to as NKX2-5 GFP iPS cells¹⁶ were cultured in Dulbecco's Modified Eagle's Medium (DMEM + Glutamax, Invitrogen) with 10% Fetal Bovine Serum (FBS; Invitrogen), 1% Non-essential amino acids, (Invitrogen) and 0.0007% (1% of a 35 ml/50 ml solution) β -mercaptoethanol (MP Biomedicals LLC, Solon,

Ohio). The NKX2-5 GFP iPS cells were created by isolating fibroblast cells from the tail tip of NKX2-5 transgenic mice and infecting the cells with the four essential pluripotent transcription factors Oct-4, Sox2, Klf-4, and c-Myc as previously described²⁹. Pluripotency of NKX2-5 iPS cells was verified by immunocytochemistry and quantitative PCR, and ability to form teratomas consisting of cell types from all three germ layers when transplanted into NOD-SCID mice¹⁶. To maintain pluripotency, media was supplemented with Leukemia Inhibitory Factor (LIF, Millipore, Billerica, MA) at 2000 U/ml. Embryoid bodies (EBs) were made via the hanging drop method (day 0)³⁰. iPSCs were harvested and resuspended in DMEM + 10% FBS (no LIF or BMP-4) at 1.6×10^4 cells/ml. This cell suspension was used to make 30 μ l hanging drops over $1 \times$ PBS in 100 mm petri dishes, resulting in initial concentration of 500 cells per drop. For the size separation experiment, additional plates of 200 and 400 cells per drop were prepared to broaden the size distribution of the total population of EBs. EBs were harvested 3 and 4 days after formation (day 3, 4), for sorting experiments based on the fluorescent reporter, NKX2-5, and sorting based on size, respectively.

MPFC Operation

EBs were imaged on the MPFC with optical configuration similar to that previously reported^{13, 17}. Briefly, simultaneous bright field and intensity images were taken with a Plan Apo VC 10 $\times$ air objective (Nikon, Melville, NY) at a resolution of 128 $\times$ 128 and pixel integration of 4, resulting in a scan rate of approximately 5.5 frames per second. The Ti:Sapphire laser (Spectra Physics, Santa Clara, CA) was tuned to 890 nm, resulting in an average power at the sample of approximately 20 mW. A 520/35 nm bandpass filter (Chroma Technology Corporation, Rockingham, VT) was used to collect GFP emission from the EBs. Power and gain settings were set such that less than 5% of pixels were saturated and background noise was kept to a minimum. Bright field and intensity images were collected using in-house developed software (WiscScan). Fluorescence and size measurements of EBs were made using ImageJ software. For analysis, background levels were identified such that less than 10% of pixels on a background image (i.e., containing no EBs) were saturated for all conditions.

Assessment for Cardiomyocyte Function and Immunofluorescence Staining

Imaged EBs were placed in an incubator overnight and analyzed for attachment on the following day. Each subsequent day, medium was replenished in the 6 well plate and the percentage of EBs displaying beating areas was recorded on day 7 of differentiation. Beating areas were recorded as binary events (1 or 0) assigned to a single EB, regardless of whether the EB contained multiple beating areas and irrespective of the size of the beating area. Immediately following observation, EBs were fixed with 4% paraformaldehyde and stained for cardiac troponin (cTnT), as a more quantitative measure of the percentage of cardiomyocytes formed per EB. The National Stem Cell Bank protocol (www.wicell.org, SOP-CH-210C) for immunolabeling of cardiac markers in EBs was utilized for this assessment. Mouse anti-troponin T (cardiac isoform Ab-1, cTnT, clone 13-11, Fisher Scientific, Pittsburgh, PA) primary antibody and goat anti-mouse Alexa Fluor 568 (Molecular Probes/Invitrogen, Carlsbad, CA) secondary antibody were used. Fluorescence images were acquired using a Zeiss Axiovert 40CFL inverted microscope (Zeiss, Germany) with a 4 $\times$ objective, using a 560/55 nm bandpass excitation filter and 645/75 bandpass emission filter. The total fluorescence intensity per EB was calculated by summing the intensity of those pixels above background fluorescence obtained using samples stained with the secondary antibody only.

Flow Cytometry Applications of WiscScan Software

All real-time particle analysis was carried out by the WiscScan acquisition software. WiscScan integrates ImageJ¹⁸, an open source, Java-based image processing program, for all image calculations. Two primary image-processing functions were implemented on particles in flow, namely size and fluorescence intensity per unit area. A results table is displayed during an acquisition trial recording the optical imaging plane in which a particle is detected, the corresponding particle area (size), number of intensity pixels above background, the mean intensity of pixels above background (intensity), and the total intensity divided by particle area (intensity per unit area). The user can then define parameters on which to sort, by checking the corresponding box in WiscScan.

To begin acquisition, the background intensity applied to unmodified images, as well as the minimum particle size to be detected must be defined. This ensures that debris will not be detected or be sorted as a false positive event. Before performing a sorting trial, a pre-sort analysis trial is executed with a representative sample population, to acquire distributions of sample size, and intensity per unit area. From the acquired results table, the user can decide which of the parameters to sort based on, as well as the lower and upper bounds of that parameter to isolate a population of interest.

Timing

Sorting trials are performed in real-time, and so program timing becomes a critical factor to obtain high sorting efficiency. At current acquisition resolutions, WiscScan acquires image frames between 0.1720 and 0.1880 seconds per frame (5.31 to 5.81 frames per second), requiring all ImageJ calculations to be complete at least 30 ms before the next image frame is loaded, allowing for adequate time to signal the data acquisition card and sorting valves. The major tasks that must be completed within the 150 ms allowance are to display any required images from WiscScan in ImageJ, carry out appropriate image calculations, compare results with user-defined values, and signal capture. Over the course of 5000 frames (~15 minutes), measurement calculations in ImageJ were completed in an average time of 8.7 ms and 63.2 ms, for the size and intensity per unit area measurements, respectively. Thus, image processing algorithms are easily completed in the allotted 150 ms, and do not restrict the throughput of the MPFC.

Statistical Analysis

For comparison of size, NKX2-5 expression, beating areas and cardiac Troponin T expression between sorted and unsorted EB populations on day 7 of differentiation, a normal distribution was assumed and one-way analyses of variance (ANOVA) and Student *t*-test for unpaired samples were used. Data were reported as average $\pm$ standard deviation and were analyzed with JMP 5.0.1 for Windows (SAS Institute, Inc., Carey, NC). A 95% confidence ($P < 0.05$) interval was applied for statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of new WiscScan functionalities for the MPFC. A) Images demonstrating the sequence for real-time image segmentation including (from left to right) raw bright field image, “Find Edges”, “Gaussian Blur” filter with variable sigma radius (controlled by user), applied autothreshold feature (minimum method) with “Erode” feature, and final region of interest (ROI). B) Images demonstrating the sequence for overlaying the particle ROI onto the intensity image including (from left to right) raw intensity image, background-corrected image, the final ROI acquired from the bright field image, the final intensity image per bright field area, with all pixels excluded that are not contained within the ROI, scale bar = 200 μm .

Figure 2.

Sorting EBs based on area measurement. A) Schematic of experimental protocol. EBs were made in hanging drops with starting concentrations of 200, 400 or 500 cells per 30 μl drop. EBs of all sizes were harvested and mixed together at day 4. EBs greater than 330 μm in diameter were isolated (Large Fraction) first while those less than 330 μm were bypassed and collected in the main outlet port. The population collected in the main outlet port was then sorted by setting a sorting threshold of 250 μm in diameter. Next, the positively sorted fraction consisted of those EBs above 250 μm in diameter (Medium Fraction), while those less than 250 μm were bypassed and collected in the main outlet port (Small Fraction). EBs were maintained separately and plated onto 6-well plates. They were then assessed for beating areas and stained for cTnT on day 7 of differentiation. B) Histogram depicting the size distribution of the EBs to be sorted on the MPFC. Thresholds for positively selecting the large population (solid line) and medium population (dotted line) are displayed. C) Bright field images taken in flow of representative EBs from small, medium and large fractions separated during the consecutive sorting trials, scale bar = 200 μm . D) Sorting efficiencies and enrichment ratios for purifying the large population (n=3 trials, 184 EBs) and the medium population (n=3 trials, 81 EBs). Error bars are standard deviation of trials.

Figure 3.

Sorting EBs based on NKX2-5 expression with the MPFC. A) Schematic depicting the experimental protocol for sorting trials. EBs were harvested on day 3 of differentiation and subsequently analyzed on the MPFC without sorting to determine the distribution of NKX2-5 expression of the population. Thresholds were set such that EBs exhibiting the top 25% fluorescence intensity per unit area were positively selected and sorted. EBs are collected from the sorting and main outlet ports and plated separately into 6-well plates to attach and continue to proliferate and differentiate. On day 7, EBs were analyzed for beating areas and stained for cTnT expression. B) Distribution of normalized NKX2-5 expression for the sample input population of EBs as determined in a pre-sort analysis trial. C) Bright field and intensity images of an EB determined to exceed the user-defined sorting threshold (top row) and an EB less than the sorting threshold value (bottom row). The processed intensity image illustrates the ROI overlaid onto the intensity pixels above background in real-time. The total intensity of all the pixels above background within the ROI was divided by the pixel area of the ROI for a final measurement of fluorescence intensity per unit area, scale bar = 200 μ m. D) Sorting efficiency and enrichment ratios determined from sorting trials (n=3 trials, 226 EBs). Error bars are standard deviation of trials.

Figure 4.

Capacity of sorted fractions of EBs to develop cardiomyocytes A) Average size of resulting EB populations (small, medium and large) collected after sorting with the MPFC. B) Beating areas and corresponding cTnT expression of populations sorted based on size. C) Average normalized NKX2-5 expression of EBs collected from sorting and main outlet ports, determined by static imaging after sorting. D) Beating areas and corresponding cTnT expression of EB populations sorted based on NKX2-5 expression. E) Scatter plot of EB diameter and NKX2-5 intensity of EBs from all replicates. Each dot corresponds to a single EB and the darker box corresponds to the sorted population. F) Beating areas and corresponding cTnT expression of EB populations sorted based on EB diameter and NKX2-5 expression according to the thresholds set in the scatter plot (E). Brackets indicate significant differences ($P < 0.05$).