

Rapid, label-free counting and characterization of live embryoid bodies

Introduction

Embryonic stem (ES) and induced pluripotent stem (iPS) cell colonies spontaneously differentiate and form three-dimensional multicellular aggregates, called embryoid bodies (EBs), when cultured in suspension without key stem cell growth factors. EB formation is a common intermediate during the *in vitro* differentiation of ES/iPS cells into specialized cell types. The size distribution of EBs plays a significant role in the efficiency of differentiation and in production yields (Dang et al. 2004; Ng et al. 2005; Falconnet et al. 2006).

Cultures maintained by enzymatic passage result in formation of a heterogeneous EB population, varying in size and morphology, and in the ultimate yield of differentiated cells. Recent efforts to enrich EBs of preferred size to increase differentiation potential have included biocompatible coatings (Valamehr et al. 2008), microcontact printing (Bauwens et al. 2008), and forced aggregation systems (Burrige et al. 2007) with some success. The EB Generation Application on LEAP™ (Cyntellect, Inc.) was recently introduced (See Application Note: Automated Embryoid Body Generation powered by LEAP™ – Generation of EBs of Specific Size), enabling the automated generation of EBs of specific sizes. These developments for improved EB generation have led to a need for routine quality assessment of EB number, size, and shape.

The Celigo™ Colony Counting: Embryoid Body Application quickly and accurately analyzes EB populations of all shapes and sizes, and does so in a non-destructive manner so that the EBs analyzed can be subsequently used. Current methods typically require acquisition of hundreds of images on a standard microscope followed by manual analysis or loading into another software program which provides only basic measurements (e.g., EB diameter). Because these analyses are difficult and slow, thorough characterization of EBs prior to differentiation is often omitted. A fast, automated method for EB analysis greatly reduces the time and effort needed, and allows quality control of EBs prior to investing in long-term differentiation cultures. In addition, EB characteristics which enhance particular differentiation pathways can be identified to improve differentiation efficiency and reduce culture costs.

The Colony Counting: Embryoid Body Application uses Celigo's large area rapid scanning capability to count and determine the size, shape, and morphology of EBs. The system records whole well images of multi-well (384W to 6W) plates enabling the tracking of live EB characteristics for correlation with final differentiation patterns.

Approach and Results

Human iPS cells (Kan and Mercola 2009) were cultured in normal growth medium on murine embryonic feeders (MEFs) in 96-well plates to confluence. EBs were generated using the Automated EB Generation Application and EB Generation Kit on LEAP (Cyntellect, Inc.). This application systematically sections confluent stem cell cultures into defined sizes using a laser. After sectioning cultures into specific sized clumps, the cultures were treated with collagenase for 1 hr to dislodge the clumps which were then transferred to low attachment

cell culture plates in differentiation medium (80% KODMEM, 20% FBS, 1% Glutamax, 1% NEAA, 0.1 mM BME). Well-defined EBs formed within a few days of culture. EBs generated by standard methodology (collagenase passaged iPS cell cultures treated with collagenase for ~1 hr to lift colonies) were used for comparison.

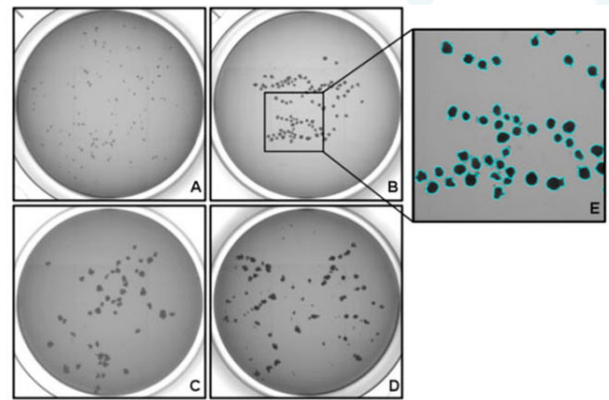
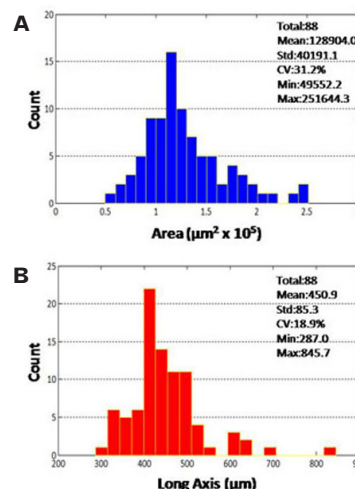


Fig. 1. EB cultures in 12W plate imaged using Celigo's Colony Counting: Embryoid Body Application. (A-C) Resulting EB populations generated from 400 μm, 800 μm, and 1600 μm clumps using the EB Generation Application on LEAP, respectively. (D) Resulting EB population generated from collagenase passaged iPS cell cultures. (E) Zoomed in image of EBs shown in B, segmented using the EB Application Application.

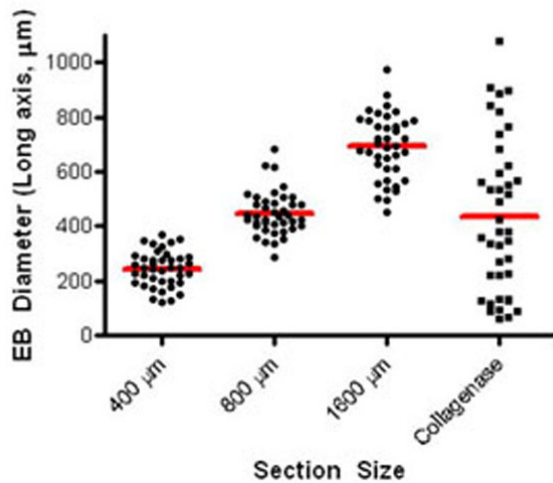
EBs were analyzed on day 4 of culture using Celigo's Colony Counting: Embryoid Body Application, acquiring brightfield images of EBs growing in 12 well plates (Fig. 1A-D). The Colony Counting Application was then used to segment the border of each EB within the population (Fig. 1E). Using segmentation features, small debris can be easily removed and touching EBs can be separated.



The Colony Counting: Embryoid Body Application includes analysis of the number of EBs per well as well as the size and shape of the EB population. The mean, standard deviation, minimum, maximum and distribution of each feature is available (Fig. 2).

Fig. 2. The Colony Counting: Embryoid Body Application was used to characterize the EB population shown in Fig 1B. The Application analyzes the total number of EBs/well and the size and shape.

Celigo's Colony Counting: Embryoid Body Application was used to analyze EB populations generated with the LEAP EB Generation Application and from collagenase-passaged cultures. EBs generated using the collagenase-passaged iPS cell cultures formed highly variable sized EBs. In contrast, EBs generated using the LEAP EB Generation Application were significantly more uniform in area, diameter (long and



	Area (μm ²)	Long Axis (μm)	Short Axis (μm)	Perimeter (μm)
400 μm	34272 (41)	245 (26)	179 (21)	834 (23)
800 μm	126810 (30)	448 (18)	362 (16)	1632 (15)
1600 μm	288667 (40)	702 (19)	531 (23)	2376 (21)
Collagenase	139998 (99)	435 (65)	312 (61)	1440 (62)

short axis), and perimeter (Fig. 3).

Fig. 3. Analysis of EB populations shown in Fig 1 using Celigo's Colony Counting: Embryoid Body Application. EBs generated using the EB Generator Application on LEAP results in more uniform EB populations of specific sizes as compared with EBs derived by standard methodology (n=40 EBs/population).

Conclusions

The Colony Counting: Embryoid Body Application on Celigo provides an efficient, reproducible, automated method for assessing the number, size, and shape of live EBs within multi-well plates. Tracking EB characteristics in culture allows correlations to be drawn between EB size and shape with differentiation potential. Identification of EB population characteristics necessary for differentiation should dramatically increase the efficiency of forming differentiated cell types from ES/iPS cell cultures. In addition, the ability to non-destructively monitor large EB populations over time should help define necessary growth characteristics for large scale differentiation procedures, particularly those involving the use of bioreactors.

References

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- Burridge et al., Stem Cells 2007, 25:929.
- Dang et al., Stem Cells 2004; 22:275.
- Falconnet et al., Biomaterials 2006, 27:3044.
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- Ng et al., Blood 2005; 106:1601.
- Valamehr et al., PNAS 2008, 105:14459.

About the Celigo Adherent Cell Cytometer

The Celigo adherent cell cytometer enables *in situ* brightfield and fluorescence analysis of adherent cells with minimal sample manipulation. The system allows for the measurement of multiple cell-based parameters. The system enables cell biologists to:

- Analyze cell cultures *in situ*, with minimal disturbance
- Eliminate invasive enzymatic disruption during sample preparation
- Image cells in brightfield and fluorescence on the same platform
- Analyze every cell in every well with no 'edge effect'

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