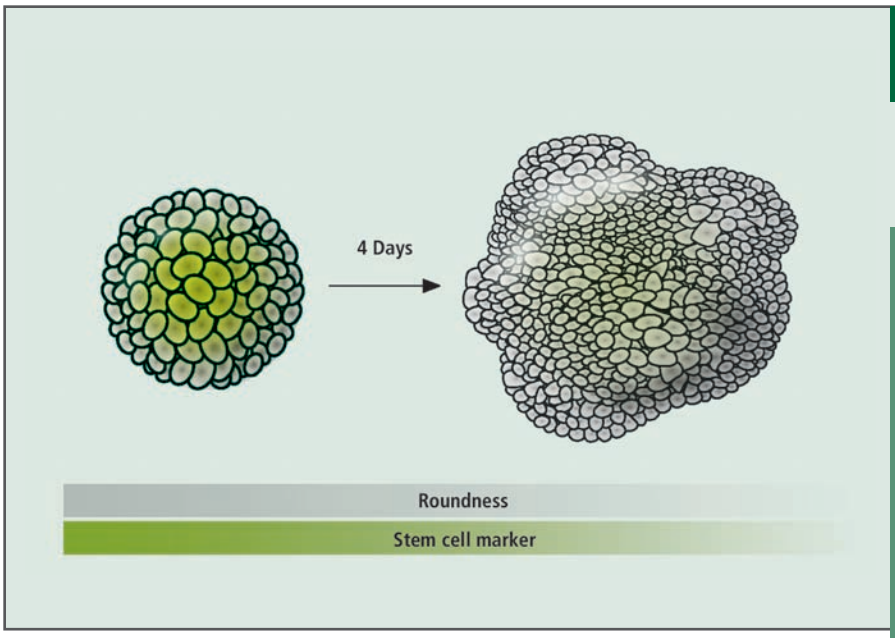

APPLICATION NOTE

Quantitative Analysis of Embryoid Bodies Using the Operetta System

Key Features

- 3D confocal imaging
- Cell identification in maximum intensity projection image
- Quantification of marker positive cells

Identification of Differentiating Area

Background

Embryoid bodies (EBs) are spherical three-dimensional cell aggregates derived from embryonic stem cells (ESCs) which serve as model systems for embryonic development. The formation of EBs allows the study of cellular and molecular interactions in a three-dimensional manner in the early stages of embryogenesis. The differentiation process results in a large variety of differentiated cell types that can be studied and characterized in vitro [Desbaillets *et al.,* 2000; Itskovitz-Eldor *et al.,* 2000; Son *et al.,* 2011].

High content imaging enables quantitative analysis of fluorescent marker intensities combined with analysis of cellular morphology and textures, and has proven to be a precise and rapid tool for characterizing cells. Here, we present an imaging approach for cell identification and stem cell marker quantification in a three-dimensional object using the Operetta® High Content Imaging System and the Harmony® High Content Imaging and Analysis Software. Classification of stem cell marker positive and negative cells allowed the discrimination between differentiated and undifferentiated areas of the EBs.

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Application

ESCs were used that express GFP under the control of the Oct4 promoter, which directs transcription of a well known stem cell marker [Rodriguez *et al.,* 2007]. To study the differentiation process, ESCs were differentiated into EBs using the hanging drop method. ESCs were counted and adjusted to $3x10^4$ cells/mL in ESC medium without the cytokine LIF, and 20 µL drops were spotted on the lid of a bacterial dish. The lid was inverted and put back on the dish, which was filled with PBS to prevent desiccation. After 4 days of incubation in hanging drops, EBs were collected and propagated in ESC medium without LIF. EBs were collected at day 4 and day 8, washed with PBS and fixed with 3.7% formaldehyde for 30 min. Fixed 4-day and 8-day old EBs were permeabilized with 0.5% Triton® X-100 (15 min). The nuclei and actin cytoskeleton were stained by incubation with Hoechst 33342 (20 µM) and Rhodamine Phalloidin (Invitrogen®, final conc. 1 unit/mL), respectively. Finally the EBs were transferred to a 384-well CellCarrier™ microtiter plate (PerkinElmer, 6007550) for imaging.

Images were acquired on the Operetta system using the confocal mode and a 20 X high NA objective. A z-stack of 80 planes with a total distance of 160 µm was measured. For image analysis, the stack rendering method "maximum intensity projection" was used. With this method all planes of the stack are reduced into a single projection image. This image is created by selecting pixels with the highest signal intensity at each position across the z-stacks and combining them into a single image. The maximum projection images (Figure 1) were used to calculate the morphology and intensity properties of the 4-day and 8-day old EBs.

For image analysis, the EB region could be clearly discriminated from the background by texture-based segmentation using the actin image. By calculating the morphology properties of the 4-day old and 8-day old EB regions, there was a significant difference in the readout comparing area and roundness of the bodies. While the total area of the 8-day old EB clearly increased, the roundness decreased due to an uneven and complex structured surface of the EB (Figure 3). The Hoechst channel enabled further segmentation of the EB into single nuclei. By calculating the Oct4 intensity properties of each nucleus, cells were subdivided into Oct4 intensity classes, allowing the generation of a histogram (Figure 3). The histogram clearly shows that the largest proportion of cells from the 4-day old EB show a high Oct4 intensity, while the 8-day old EB contains mainly cells with lower Oct4 intensities. This provides further evidence of the advanced differentiation of the growing EB. Setting an Oct4 intensity threshold allowed the separation of the cells into marker positive and marker negative cell populations, enabling the identification of differentiated and undifferentiated regions inside the EB (Figure 2).

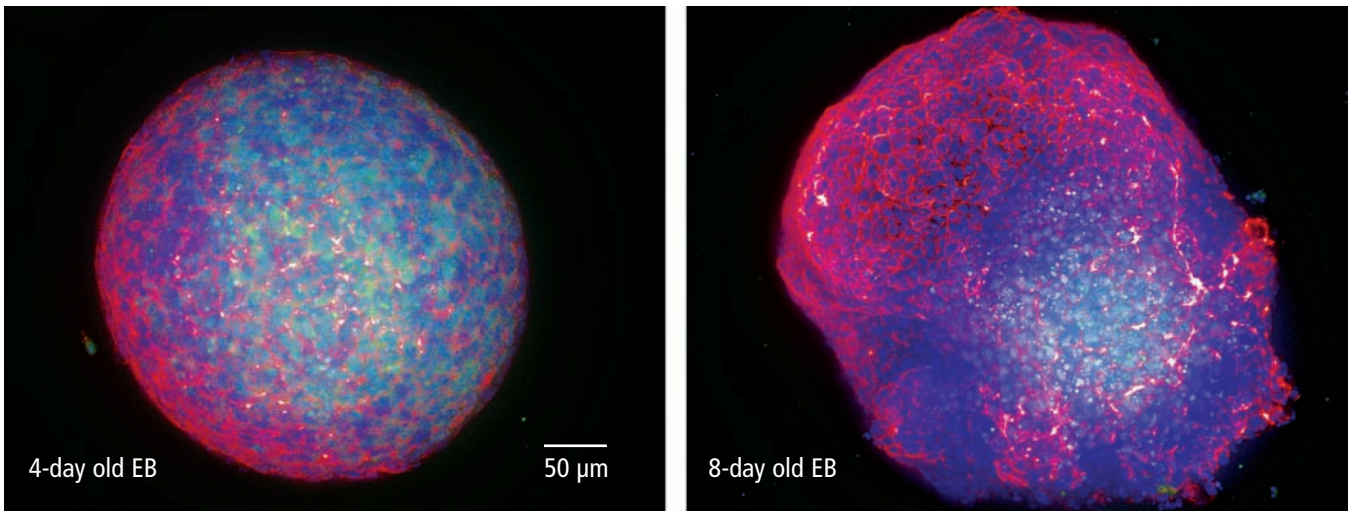


Figure 1. Maximum intensity projection images of a 4-day old EB (left) and 8-day old EB (right). The maximum intensity projection was generated using all 80 planes of the acquired z-stack. Images show a false color overlay of nuclei (blue), actin (red) and Oct4 (green). Compared to the 4-day old EB, the 8-day old EB is larger, more complex and expresses less stem cell marker Oct4, indicating advanced differentiation.

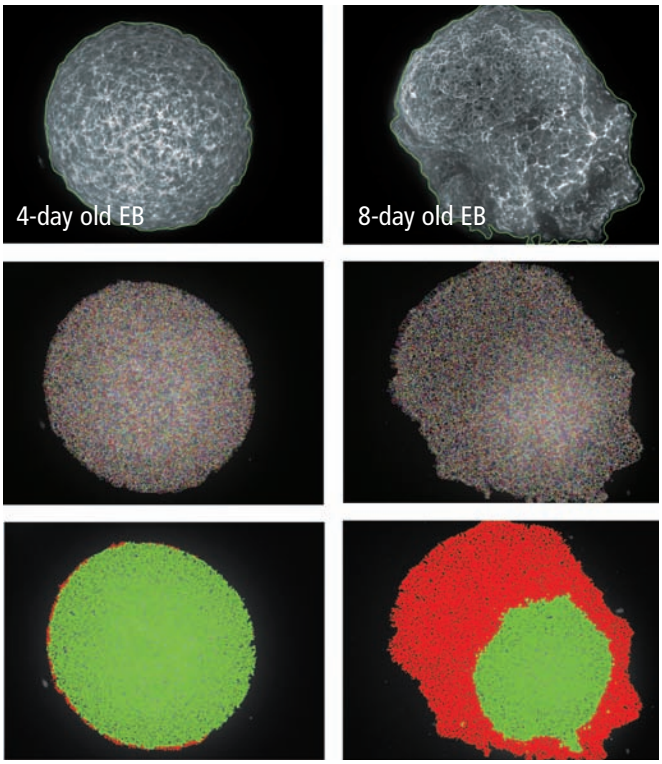
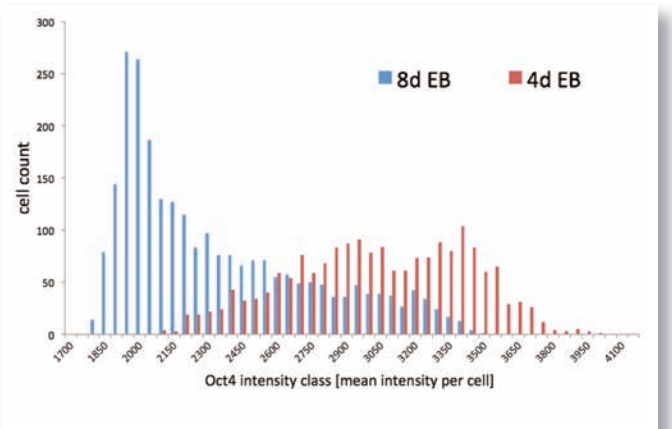


Figure 2. Image-based segmentation and identification of Oct4 positive/negative areas in the maximum intensity projection image. Texture-based segmentation enabled the EB region (upper panels, green border) to be found. A nuclear mask was generated in the EB region and was used to calculate the intensity properties of each nucleus in the Oct4-channel (middle panels). Setting an intensity threshold allowed the selection of a marker positive and a marker negative cell population (lower panels), and an undifferentiated area (shown in green) and a differentiating area (shown in red) could be located.



	EB 4 days	EB 8 days
Area [µm²]	146045	173542
Roundness	0.970446	0.852404
Oct4 mean intensity	2982.28	2359.58
Oct4 pos area [%]	97	35

Figure 3. Quantification of several readouts indicating differentiation of the EBs. The Oct4 mean intensities of all detected nuclei were subdivided into intensity classes. The histogram (upper panel) shows the number of nuclei in each of the intensity classes for both EBs. The 4-day old EB (red bars) mainly comprises cells with high Oct4 intensity whereas the 8-day old EB (blue bars) contains mainly cells with low Oct4 intensity. The table (lower panel) summarizes a selection of the most important quantitative readouts for evaluating the progress of EB differentiation. As the increased area of the 8-day old EB indicates growth, the decreased values for roundness and the Oct4 positive area both imply differentiation of the EB.

Conclusion

In this study, we analyzed three-dimensional embryoid bodies (EBs) using the Operetta High Content Imaging System. The applied method of confocal stack measurement combined with analysis of the maximum intensity projection image is a robust approach for characterizing 3D bodies. The maximum intensity projection comprises extracted information from all planes rather than each plane individually so that image analysis can be achieved much more quickly.

Alongside the quantification of area, roundness and Oct4 intensity, the Harmony High Content Imaging and Analysis Software enables the discrimination between differentiated and undifferentiated regions of the EB by classifying cells according to their Oct4 expression level. For the EBs studied here, we observed a differentiation in the rim and outer regions while the center remained undifferentiated after 8 days of incubation. Further characterization of the differentiation process through the inclusion of additional differentiation markers into the high content imaging approach would be straightforward.

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