CURIOSIS

APPLICATION NOTE

Assessment of adipogenesis degree in real time

Using Celloger® Pro

■ Introduction

Adipocytes serve as crucial energy reservoirs, efficiently converting surplus calories into fat for future energy needs. However, excessive fat accumulation can lead to obesity, a condition associated with various health problems in our modern society.[1] Beyond their role in metabolism, adipocytes also influence immune responses and reproductive functions[2] through hormone utilization such as leptin and adiponectin.

The process of adipogenesis, where preadipocytes differentiate into mature adipocytes, varies across species. Additionally, the proportion of cells undergoing adipogenesis differs between in vivo tissues and in vitro cell culture plates.[3] Researchers actively investigate the factors involved in adipogenesis and the intricate regulatory mechanisms underlying this complex process.

In a review conducted by Sylvia P. Poulos, the differentiation ratio of cells varies depending on the components of the culture media. Additionally, the expression levels of transcription factors associated with adipogenesis change over time, whether during seeding or post-confluence. Therefore, assessing the degree of adipogenesis at different time points is crucial. The use of automated live-cell imaging equipment, such as the Celloger® Pro, makes it convenient to monitor and measure adipogenesis. In this application note, we introduce methods for assessing adipogenesis during the differentiation process using the widely studied preadipocyte cell line, 3T3-L1.

■ Materials and Methods

Cell maintenance and cell confluency inspection

3T3-L1 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium with high glucose, Welgene, LM001-10) supplemented with 10% BCS (Bovine Serum, Gibco, 26170043) and 1% P/S (Penicillin-Streptomycin, Gibco, 15140122). When the cells reached 80~90% confluence, they were sub-cultured. To induce differentiation, cells were seeded in a 12-well plate at concentrations of $1X10^4$ cells/cm^2, $5X10^3$ cells/cm^2, $2.5X10^3$ cells/cm^2, and $1.25X10^3$ cells/cm^2. After seeding, the culture medium was changed on the second day, and then on the following day, the cell density was measured using the Celloger® Pro with a 4X lens.

Adipogenesis induction using differentiation media (DM) and observation of cell morphology during adipogenesis

On the second day after achieving confluency, we introduced DM I, which consists of DMEM supplemented with 10% FBS (Fetal Bovine Serum, Welgene, S001-01), 1% P/S, 1 μg/ml Insulin (Insulin solution human, Sigma, I9278-5ML), 0.25 μM Dexamethasone (Sigma, D2915-100MG), and 0.5 mM IBMX (3-Isobutyl-1-methylxanthine, Sigma, I5879-100MG). After an additional two days of cultivation, we switched to DM II, composed of DMEM supplemented with 10% FBS, 1% P/S, and 1 μg/ml Insulin. To assess the impact of Rosiglitazone on adipogenesis, we added 2 μM Rosiglitazone (Sigma, R2408-10MG) to both DM I and DM II. Throughout the differentiation period, cell images were captured at one-hour intervals using the Celloger® Pro equipped with 4X or 10X lenses.

Quantification of lipid droplet using fluorescence

During the differentiation process, cells were cultured with 1 μM LipiDye II (FDV-0027, Funakoshi) to fluorescently observe lipid droplets. The coverage (%) of green fluorescence was measured using the Celloger analysis software to calculate the area of the lipid droplets. To enhance the accuracy of assessing the uneven occurrence of adipogenesis, five randomly selected points per well were imaged, and the average value was calculated.

■ Result

Observing morphological changes during differentiation

The most significant structural change during adipocyte differentiation is the formation of lipid droplets. These are organelles enveloped by a monolayer of phospholipids, containing triglycerides and sterol esters. Adipocytes are classified into brown, beige, and white cells, each with unique characteristics. Unlike beige and brown cells, which typically contain multiple lipid droplets per cell, white cells are characterized by a single, large lipid droplet that occupies a significant portion of the cytoplasm, making them easily distinguishable from other cell types (Figure 1).

These three types of adipocytes have distinct functions. White adipose tissue (WAT), prevalent in obesity, primarily serves as an energy storage site, while brown adipose tissue (BAT) aids in energy expenditure through thermogenesis. Recent studies have highlighted that Batokines, factors released by BAT, can impact various organs[4], thereby influencing overall metabolism.[4, 5] Additionally, beige fat cells, situated within WAT, exhibit traits similar to those of BAT, including thermogenic capabilities. Moreover, these cells can undergo transdifferentiation from mature WAT[6], attracting significant attention from many researchers.

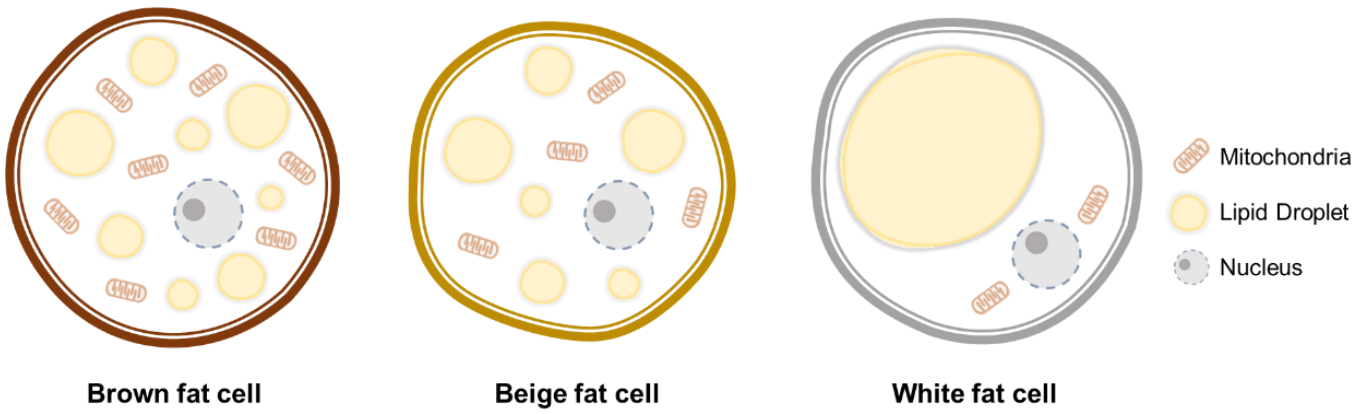

Figure 1. Three types of adipocytes

We induced adipogenesis in 3T3-L1 cells and observed changes in cell morphology at one-hour intervals throughout the process using the Celloger® Pro. Figure 2 depicts the differentiation process of 3T3-L1 cells over time. While the timing of lipid droplet formation varied depending on cell passage, in most cases, it was promptly observed following the introduction of DM II containing Rosiglitazone. The cells predominantly evolved into brown fat-like cells with numerous tiny lipid droplets. Using the Celloger® Pro with a 10X lens, we could distinctly identify lipid droplets over 3 μm in diameter as clear, water droplet-shaped figures, eliminating the need for further staining.

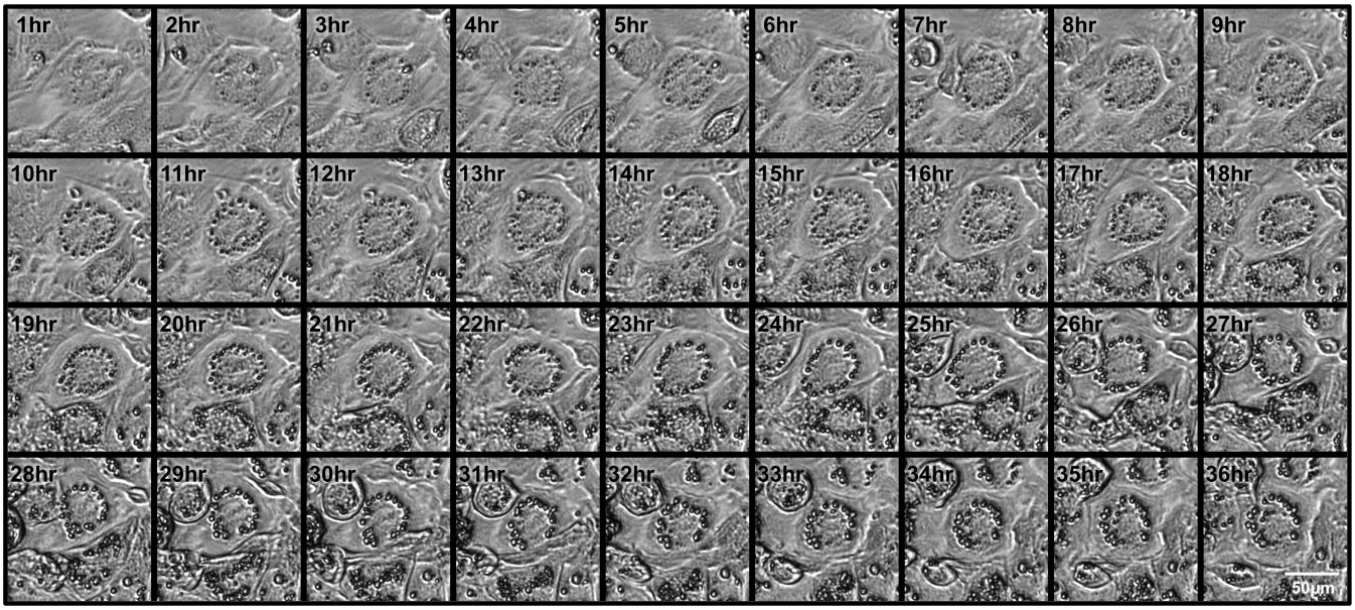

Figure 2. Time-lapse sequence of adipocytes during differentiation

This sequence presents images captured at one-hour intervals from 1 hour to 36 hours after treatment with DM II and Rosiglitazone. (using a 10X magnification, cropped images)

Quantification of lipid droplet in real time

Many researchers use Oil Red O reagent to assess the level of differentiation by evaluating the extent of lipid droplets. Oil Red O is a dye commonly used to stain lipid-rich structures such as lipid droplets, enabling visualization under a microscope. However, staining is not feasible under physiological conditions, making it difficult to track changes over time. Therefore, we chose LipiDyeII, a fluorescent dye suitable for live cells. LipiDyeII not only stains lipid droplets but also allows for analysis in live cells, making the process straightforward. Cells in DM containing LipiDyeII exhibited a gradual manifestation of lipid droplets, characterized by green fluorescence. The fluorescent signals precisely coincide with the enveloped structures observed in the bright field image (Figure 3). Even when lipid droplets are obscured within cell structures (indicated by arrows in Figure 3) or are too small to distinguish in bright field (depicted by the dotted circle in Figure 3), they can be readily identified using fluorescence.

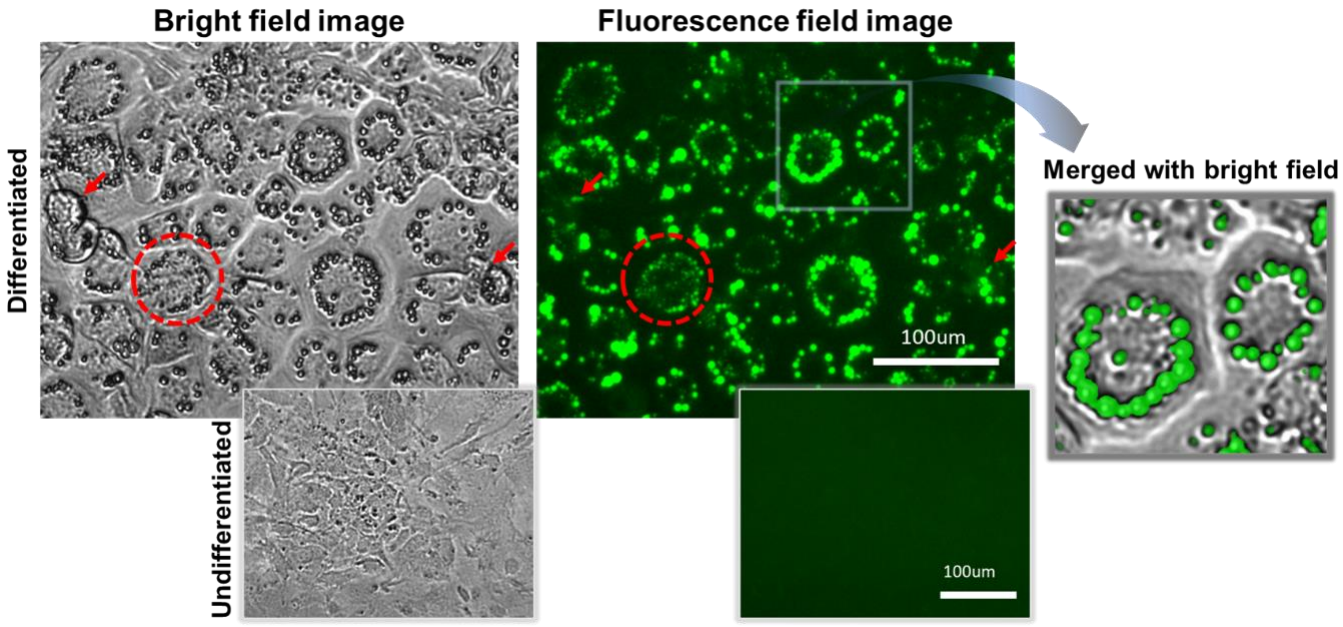

Figure 3. Observation of adipocyte with lipid droplet in bright field and fluorescence field

Images were captured 24 hours after replacing DM II containing Rosiglitazone and treating with LipiDyeII using Celloger® Pro with a 10X lens.

We quantified the differentiation ratio by measuring the fluorescence coverage, which indicates the proportion occupied by lipid droplets (Figure 4). Since differentiation does not occur uniformly across the entire plate but rather in patches, it is necessary to examine a wide area for comprehensive analysis. However, using low magnifications such as 2X is insufficient for accurately measuring small lipid droplets. Therefore, by utilizing a 4X magnification and the multi-position imaging function to scan multiple points, we were able to measure the proportion of lipid droplets over a slightly wider range and obtain more reliable data.

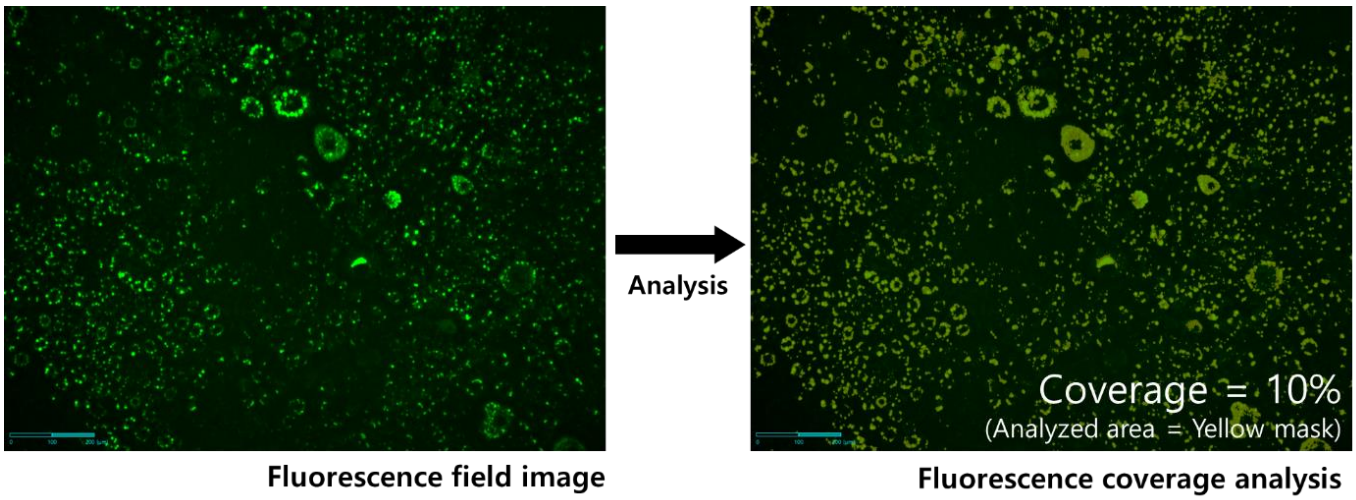

Figure 4. Measurement of lipid droplet area using Celloger analysis software

The image was captured using the Celloger® Pro with a 4X lens on the second day after treatment with DM II and LipiDyeII. Fluorescence area was distinguished using the **adaptive threshold method* and calculated in the Celloger analysis software.

**Adaptive threshold method: an image processing technique that calculates varying threshold values for different regions of an image based on local pixel value, enabling effective segmentation in images with uneven illumination or contrast.*

Assessment of differentiation level based on cell density and the Rosiglitazone administration

The adipocyte differentiation process is highly regulated and influenced by numerous factors, including the preadipocytes' environment and pharmacological interventions.[7, 8, 9] Among these, cell confluency at the time of induction of differentiation is recognized as a crucial parameter that affects the efficiency and quality of adipocyte formation.[10] Furthermore, pharmacological agents such as Rosiglitazone, a potent agonist of peroxisome proliferator-activated receptor gamma (PPARγ), have been demonstrated to play a vital role in modulating adipogenesis.[11, 12, 13, 14, 15] PPARγ is a key transcription factor that drives the expression of various genes involved in adipocyte maturation and function.

To understand the interplay between cell confluency and Rosiglitazone treatment in the context of adipocyte differentiation, we evaluated the degree of differentiation following the experimental scheme as described in Figure 5.

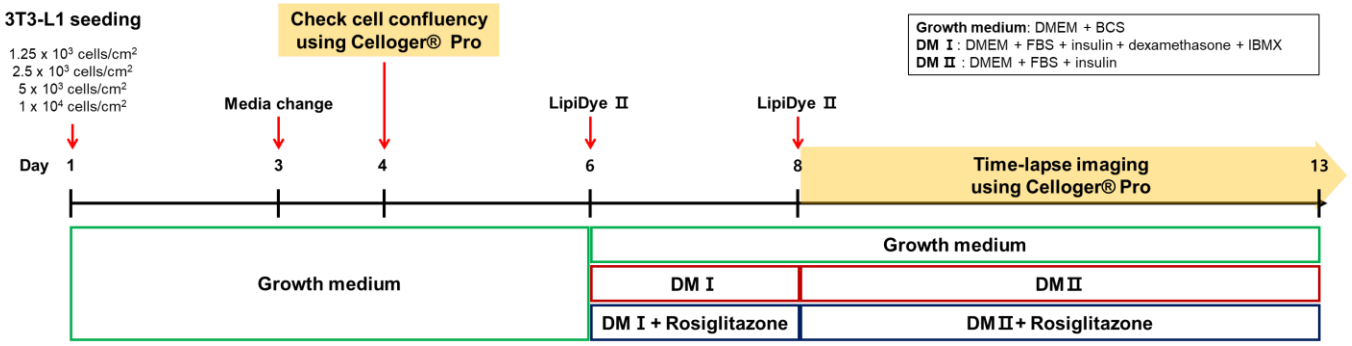

Figure 5. Experimental scheme of 3T3-L1 differentiation

To evaluate how the initial density of cells affects the differentiation of adipocytes, we seeded 3T3-L1 cells at four different densities. On the third day post-seeding, we acquired cell images to verify the level of confluency using Celloger's reservation functions, which automatically captured cell images on the designated date (Figure 6a). These captured images were then analyzed using Celloger analysis software to determine the degree of confluency (Figure 6b). This initial assessment was critical to establish a baseline for understanding the influence of cell confluency on subsequent differentiation.

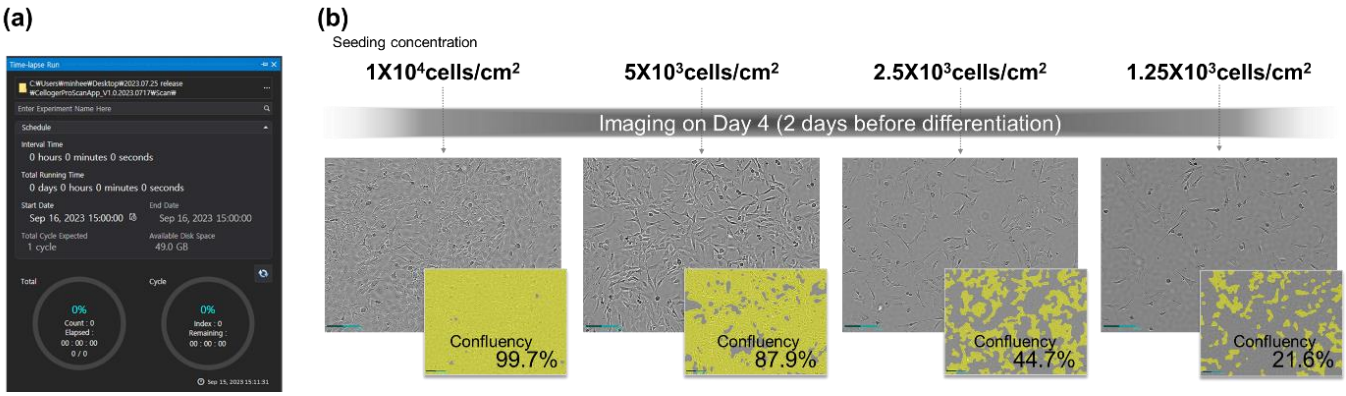

Figure 6. Confirmation of cell confluency according to cell seeding density

After seeding the cells, we assessed their confluency on the third day using Celloger's time-lapse scheduling feature. (a) Celloger scan software time-lapse schedule window (b) Images analyzing cell confluency

On day 6, two days after verifying cell confluency, we initiated the adipocyte differentiation process. To determine the impact of Rosiglitazone on this process, experiments were conducted with two groups: one treated with DM containing Rosiglitazone and the other without Rosiglitazone (Figure 5). We assessed the adipocytes' differentiation level by applying LipiDyeII dye and then measuring the fluorescent coverage, which indicates the lipid droplet area. This measurement was conducted at five different positions using the Celloger analysis software. Figure 7 presents the results observed on the day 13 (Figure 7a) and over the entire 5-day period (from day 8 to day 13) (Figure 7b) after addition of DM II with or without Rosiglitazone. The administration of Rosiglitazone markedly augmented the rate of adipocyte differentiation, as indicated by the enhanced fluorescence area denoting increased lipid accumulation. Cells maintained in DM II without Rosiglitazone manifested a differentiation rate of merely 4.7% after 120 hours. Conversely, culturing cells in DM II with Rosiglitazone led to a rapid increase in differentiation, reaching a cell differentiation percentage of 16.6% within 97 hours. However, there was little difference in the degree of differentiation based on cell concentration.

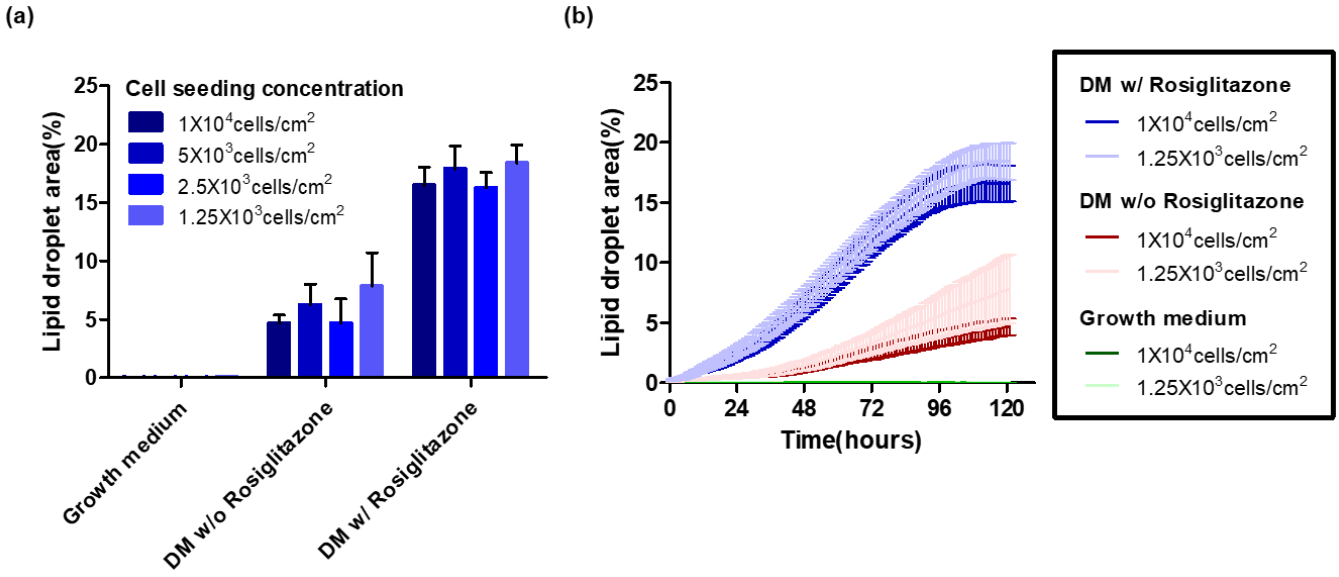

Figure 7. Quantification of lipid droplet area depending on cell density and the Rosiglitazone administration

(a) The bar graph illustrates the degree of adipogenesis depending on cell density on day 13. Error bars represent the standard error (n=5). (b) This graph shows the progression of lipid droplet formation over time after the addition of DM II (from day 8 to day 13). The analysis involved capturing fluorescent images at five different locations, with standard errors depicted by shaded areas in pink or sky-blue (DM w/ Rosiglitazone: n=5, DM w/o Rosiglitazone: n=5, Growth medium: n=1). Cells treated with DM II containing Rosiglitazone exhibited a significantly faster increase in differentiation compared to those without Rosiglitazone, as indicated by a two-tailed t-test with a p-value < 0.0001. The growth medium group represents cells that were not subjected to DM treatment. To prevent graph complexity, other concentrations were omitted.

■ Conclusion

Adipogenesis is a complex process regulated by the expression levels of various factors, often temporally controlled or influenced by interactions with other cellular elements. Understanding these intricate mechanisms necessitate meticulous observation over extended periods. Traditionally, researchers have assessed the expression levels of specific markers at certain time points during differentiation. However, the Celloger® Pro system has revolutionized this approach by allowing continuous, long-term monitoring of these pathways. With its time-lapse scheduling feature, researchers can conduct prolonged observations without time constraints, thereby enhancing experimental efficiency. A notable feature of Celloger® Pro is its adjustable magnification levels, enabling high-magnification observation of lipid droplet formation, while lower magnifications capture a broader image area to evaluate overall adipocyte differentiation. Additionally, by fluorescently staining lipid droplets and utilizing Celloger's analysis software to measure fluorescence intensity, researchers can quantify the degree of differentiation, providing numerical values to the observed changes. This combination of features offers invaluable insights into the dynamic regulatory mechanisms governing adipocyte differentiation, advancing our understanding of adipogenesis and its critical role in metabolic health.

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