Development of a digital analysis system for a novel 3D culture-based colony formation to detect malignantly transformed cells in human cell-based therapeutic products

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Abstract

The presence of malignantly transformed cells in human cell-based therapeutic products (hCTPs) is a significant safety concern. Although such cellular impurities in hCTPs can be assessed by detecting anchorage-independent growth using conventional soft agar colony formation (SACF) assays, the sensitivity of these assays is often insufficient. To overcome this limitation, we previously developed a novel tumorigenicity-associated testing method, the digital SACF (D-SACF) assay, which combines a partitioned culture of test cells to concentrate target cells with colony detection via image analysis. However, conventional soft agar culture involves complicated operations, such as preparing multilayered culture media and temperature control, and further technical optimization is required for the widespread adoption of the D-SACF assay. In this study, we focused on a new culture system incorporating a three-dimensional (3D) culture method using a liquid medium containing the low-molecular-weight agar polymer LA717 in low-adhesion culture vessels. We initially confirmed conditions for the efficient high-density 3D culture of normal cells using LA717-supplemented medium in low-adhesion 96-well plates. Using human mesenchymal stem/stromal cells (MSCs) as a normal cell model and HeLa cells as a transformed cell model, we demonstrated that the new 3D culture system effectively maintained the dispersion of MSCs and

prevented their aggregation, while transformed HeLa cells exhibited robust anchorage independence, thereby establishing the new liquid/low-molecular-weight agar colony formation (LACF) method as an alternative to SACF. Finally, by systematizing the digital analysis system for the LACF assay (D-LACF assay), which streamlines the overall workflow from the performance evaluation of the test method to product testing and result interpretation, the limitations of the conventional soft agar-based D-SACF assay were addressed and its practicality and utility were enhanced. This *in vitro* evaluation system is expected to provide a promising approach for improving the quality and safety of hCTPs.

Keywords:

Tumorigenicity, malignantly transformed cells**,** human cell-based therapeutic products**,** 3D culture, *in vitro* assay

Introduction

In a three-dimensional (3D) culture environment without an adhesion substrate, normal cells typically undergo anoikis, a type of programmed cell death triggered by the loss of cell–matrix interactions [1,2]. In contrast, malignantly transformed cells exhibit anchorage-independent growth, allowing for their survival, proliferation, and colony formation under such conditions. This distinct phenotype is a hallmark of malignant transformation, and serves as a critical parameter for assessing cellular tumorigenicity [3]. The soft agar colony formation (SACF) assay is regarded as the gold standard for the *in vitro* assessment of cellular anchorage-independent growth [4–6]. Over the years, this assay has been refined to fulfill specific experimental needs, resulting in various modifications that enhance its practicality and sensitivity [7]. For example, the conventional SACF assay is insufficiently sensitive to evaluate the presence of transformed cells as cellular impurities in human cell-based therapeutic products (hCTPs), which consist of large numbers of cells [8]. To overcome this problem, we established the digital SACF (D-SACF) assay as an innovative test method that integrates soft agar culture, partitioned culture in multi-well plates, and image analysis to enable the digital detection of colonies derived from trace amounts of transformed cells within a population of normal cells [9]. Using an imaging cytometer for

high-throughput image acquisition and automated analysis, this approach allows the efficient screening of large numbers of cells with high sensitivity to detect mixed transformed cells. Another advantage of the D-SACF assay is that increasing the number of sample partitions further enhances the sensitivity of the transformed cell detection. In a previous study, the detection limit of the D-SACF assay, when evaluating its performance using human mesenchymal stem/stromal cells (MSCs) as the product model and HeLa cells as the transformed cell model, was recorded as one HeLa cell in 1×10^7 MSCs, i.e., 0.00001% of the total number of cells. Furthermore, recent multisite studies have confirmed the high utility and feasibility of this test system [10]. Despite these advantages, the D-SACF assay presents practical challenges. As the number of wells and plates increases, preparing multilayered agar media with different concentrations and maintaining the required temperature becomes challenging. This procedural difficulty imposes a significant barrier to the feasibility and throughput for the large-scale application of the assay, underscoring the need for a more streamlined and optimized approach for the D-SACF assay.

To address these issues, we focused on a novel 3D culture system that utilizes a commercially available low-molecular-weight agar polymer LA717 (developed by Nissan Chemical Corporation, Tokyo, Japan)-supplemented medium combined with

low-attachment culture vessels. Previous studies have demonstrated that using liquid culture media containing LA717 and cultivating cells in low-adhesion containers allows for even dispersion of the cells throughout the culture medium, preventing adhesion and aggregation of neighboring cells, while maintaining contact between daughter cells after division [11,12]. Thus, this system provides an easier means of establishing a 3D environment than conventional soft agar preparation, allows for a high-density culture of cells, and may serve as a platform for evaluating anchorage-independent cell growth.

In this study, we used human MSCs as a model of normal cells and HeLa cells as a model of transformed cells to determine whether the novel liquid/low-molecular-weight agar colony formation (LACF) approach utilizing LA717 could effectively detect colony formation of malignantly transformed cells through anchorage-independent growth within the normal cell population. To overcome the limitations of the soft agar-based D-SACF assay and further improve its practicality and throughput, we optimized and systematized the protocol, ultimately establishing a digital analysis system for the LACF assay (D-LACF assay). Herein, we demonstrate how this system allows for the detection of malignantly transformed cells as tumorigenic impurities in hCTPs with high sensitivity and reproducibility, providing a promising new approach for the quality assessment of hCTPs.

Materials and Methods

Cells

All cell cultures were maintained in a humidified atmosphere with 5% CO_2 and 95% air at 37°C. iCell Human iPSC-derived Mesenchymal Stem Cells (Lot 105305, Fujifilm Cellular Dynamics, Madison, WI, USA) were cultured using the human Mesenchymal Stem Cell Growth BulletKit Medium (Lonza, Basel, Switzerland). A human cervical cancer cell line, wild-type HeLa (CCL-2, Lot 61647128), was obtained from the American Type Culture Collection (Manassas, VA, USA). We obtained plasmids pC13N-iCAG.copGFP, pZT-C13-R1, and pZT-C13-L1 described [12]by Cerbini *et al*. [13] from Addgene (Watertown, MA, USA) and introduced them into HeLa cells by electroporation. This approach enabled transcription activator-like effector nuclease (TALEN)-mediated green fluorescent protein (GFP) knock-in at the second intron of *CLYBLE* on chromosome 13, which is recognized as a safe harbor site. A stable GFP-expressing HeLa (HeLa-GFP) cell line was established for use in this study. Wild-type HeLa (HeLa-WT) and HeLa-GFP cells were cultured in Minimum Essential Medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) that was supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% GlutaMAX (Thermo Fisher Scientific,

Waltham, MA, USA), and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan). MSCs and HeLa cells prelabeled with CellVue Claret (CVC) dye (Sigma-Aldrich) were used in specific experiments following the manufacturer's instructions.

Novel LACF assay

We adopted a 3D culture method using a medium containing a 0.03% (w/v) aqueous solution of low-molecular-weight agar polymers, referred to as LA717, which is supplied as a 1% stock solution under the trade name SphereMax (Nissan Chemical Corporation, Tokyo, Japan), based on previous research [11] by Abe-Fukasawa et al. This culture method facilitates straightforward 3D cell culture with a standard cell culture medium augmented by a fixed amount of LA717 (manufacturer's recommended concentration: 0.03%) in low-adhesion culture vessels. In this study, cells were prepared in a prewarmed medium made up of a basal growth medium containing GlutaMAX-supplemented Dulbecco's MEM (DMEM; Thermo Fisher Scientific), prewarmed dissolved LA717, 10% FBS, 1% MEM Non-Essential Amino Acid solution (Thermo Fisher Scientific), and 1% penicillin-streptomycin to achieve a final concentration of 0.03–0.06% LA717 (w/v). The cell suspensions were adjusted to a density of 15,000–600,000 cells/mL and dispensed into 96-well flat-bottom

low-adhesion plates (PhenoPlate 384-well ULA-coated microplates, Revvity, Tokyo, Japan) at 100–400 μL (containing 10,000–60,000 cells) per well. These plates were incubated in a humidified atmosphere with 5% CO_2 at 37°C for approximately 3 weeks (19–23 days) without any media changes or addition.

Preparation of co-cultured cells: normal and reference transformed cells

In this study, MSCs were used as the normal cell model, whereas HeLa (-WT or -GFP) cells served as the reference transformed cell model. Samples were prepared by mixing these cells at a defined ratio, and the colony formation rate of individual transformed cells was evaluated under 3D culture conditions. To each well of a 96-well plate, 200 μL of medium supplemented with 0.03% LA717 was added, along with 20,000 MSCs and 0.125 to 1 HeLa cell. We employed a method in which the reference transformed cell suspension was initially prepared at a minimum concentration of 100 cells/mL using a procedure described later to minimize errors associated with repeated dilution steps. The required volume was then directly taken from this suspension and added to the MSC suspension, which had been preloaded into a reservoir. After thoroughly mixing, the suspensions were seeded into multiple wells. Additionally, when sampling a fixed volume from a low-concentration cell suspension, we considered the Poisson

distribution of the actual cell count along with its 95% confidence interval (CI). The CI for the Poisson distribution was calculated using the poisson.test function in R (version 3.3.3, R Foundation for Statistical Computing, Vienna, Austria). For instance, when spiking a suspension expected to contain 100 cells, executing "poisson.test (100, conf.level = 0.95)" in R yields a 95% CI ranging from 81–122 cells (indicating a rounded-down or rounded-up value).

Cell counting

The concentration of viable cells in the cell suspension was measured using NucleoCounter NC-202 Consistent Cell Counter (ChemoMetec, Cambridge, MA, USA) or Countess 3 FL Automated Cell Counter (Thermo Fisher Scientific). Based on these results, serial dilutions were performed to prepare suspensions of appropriate concentrations for each test. When preparing low-concentration HeLa cell suspensions at 100 cells/mL, a portion of the diluted suspension was used to reconfirm the concentration using the following method: 1.2 mL of the suspension was collected and incubated at 37°C for 15 min with 1 μg/mL Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan), 1 μg/mL propidium iodide (Dojindo Laboratories), and 1 μg/mL Calcein-AM (Dojindo Laboratories) (not added when counting HeLa-GFP), and then

dispensed into 96 wells of a 384-well plate (Corning, Corning, NY, USA) at 10 μL each. After centrifugation of the 384-well plate at 300 × g for 1 min, the presence or absence of viable cells in each well was analyzed by fluorescence imaging using the confocal imaging cytometer CellVoyager CQ1 (Yokogawa Electric, Musashino, Japan). Based on the number of negative wells (k) without cells out of the 96 wells, the average cell number (λ) per 10 μL was calculated using the formula $\lambda = -\ln(k)$, similar to determining the average copy number per partition in digital PCR [14]. The final concentration of the suspension was thus determined.

Cell staining

After 3 weeks of incubation, the cells were stained with various reagents under the following conditions. After carefully adding 20 μL of LACF assay medium (containing 0.03% LA717), warmed to 37°C with each dye reagent to each well, the plate was incubated at 37°C and 5% CO2 for at least 1 h. The final concentrations of each staining reagent after addition were as follows: Hoechst 33342 at 1 μg/mL, Calcein-AM at 1 μM, LysoTracker Red (Thermo Fisher Scientific) at 50 nM, and MitoTracker Deep Red (Thermo Fisher Scientific) at 50 nM. The images of each well were captured using an imaging cytometer. In this study, we used two different imaging devices: the

CellVoyager CQ1 (Yokogawa Electric) and the Cell3iMager duos2 (SCREEN Holdings, Kyoto, Japan). The former was primarily used for acquiring fluorescent images for analysis, while the latter was mainly employed to obtain clear brightfield images of the entire well. When fixing samples stained with reagents other than Calcein-AM, 15 µL of 16% paraformaldehyde (PFA) solution (Muto Pure Chemicals, Tokyo, Japan) prewarmed to 37°C was added to each well (with a final PFA concentration of approximately 1%). The samples were kept in the dark and allowed to stand overnight at 37°C. Subsequently, the samples were stored in the dark at room temperature. Fixed samples were imaged within 2 weeks.

Image analysis

An imaging cytometer was used to capture images of each well. Excitation light and fluorescence filters corresponding to the fluorescent proteins expressed in the cells or the added fluorescent reagents were selected and multichannel images of the entire well, including bright-field images, were obtained. Furthermore, multiple Z-stack images were acquired, and images focused on the cell colonies were selected. Automated image analysis was conducted for each well using an application integrated into the analyzer, employing an analysis script to segment colony features based on size, circularity, and

fluorescence intensity criteria. In this study, through image analysis, the cell survival status was determined based on the expression of fluorescent proteins or the staining reaction of cells to fluorescent reagents. HeLa cell-derived colonies were then defined and detected based on their size, shape, and circularity. However, in some cases, debris and other objects were detected as false-positive objects in the image analysis; therefore, the presence or absence of colonies in each well was ultimately confirmed by visual inspection based on bright-field images.

Calculation of the colony-forming efficiency (CFE) of reference transformed cells

Calculations were performed to determine the CFE (x) of reference transformed cells based on the following assumptions:

1. Cells are assumed to be individually dispersed in the suspension without aggregation.
2. Transformed cells seeded into a fixed number of wells (n) are distributed across the wells following a Poisson distribution.
3. Under 3D culture conditions, a single transformed cell in a well of a 96-well plate proliferates and forms a colony with a specific probability ($0 \leq x \leq 1$).
4. When multiple transformed cells are seeded into the same well, they each form

colonies independently with the same probability (x), without influencing one another.

Using these assumptions, the relationship among the number of fractionated wells (n), the average number of transformed cells per well (λ), the total number of wells with colonies (y), and the probability of colony formation (x) is described by the following equation (see Supplementary Figure 1 and 2 for detailed mathematical derivation and application examples):

$$y = \sum_{k=1}^{n\lambda} \left(n \cdot \frac{\lambda^k \, e^{-\lambda}}{k!}\right) \cdot \left(1 - (1 - x)^k\right)$$

To determine x, the upper limit of k was estimated based on the actual number of fractionated wells (n) and the average number of transformed cells per well (λ). As shown in Supplementary Figure 1, the value of k was set to a level that ensures the number of wells with colonies is not zero, regardless of the CFE, based on the seeding conditions (particularly n and λ) of the reference transformed cells. Calculations for x were performed using the Goal Seek function (What-If Analysis) in Microsoft Excel (Microsoft, Redmond, WA, USA) to obtain approximate results.

Statistical analysis

Statistical analyses were performed using Prism 10 software (version 10.3.0; GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$. The effects of culture conditions and independent test trials on the mean CFE of HeLa-GFP cells under different culture conditions were evaluated by one-way repeated measures analysis of variance (ANOVA). CFE values are presented as the mean ± standard deviation (SD) or with their corresponding 95% CI.

Results

LA717 enables stable and dispersed 3D MSC cultures at a high cell density

To evaluate the effect of LA717 on the high-density 3D culture of MSCs, cells were seeded at various densities (10,000–60,000 cells/well) in low-adhesion culture plates. In the absence of LA717, MSCs exhibited rapid aggregation, forming dense cell clusters within the first day of culture, regardless of the initial seeding density (Figure 1, left panel). This aggregation persisted and became more pronounced over time, especially in the wells with higher cell densities. In contrast, the addition of 0.03% LA717 to the culture medium significantly suppressed MSC aggregation. The cells remained largely dispersed across all seeding densities even after 14 days of culture (Figure 1, right panel).

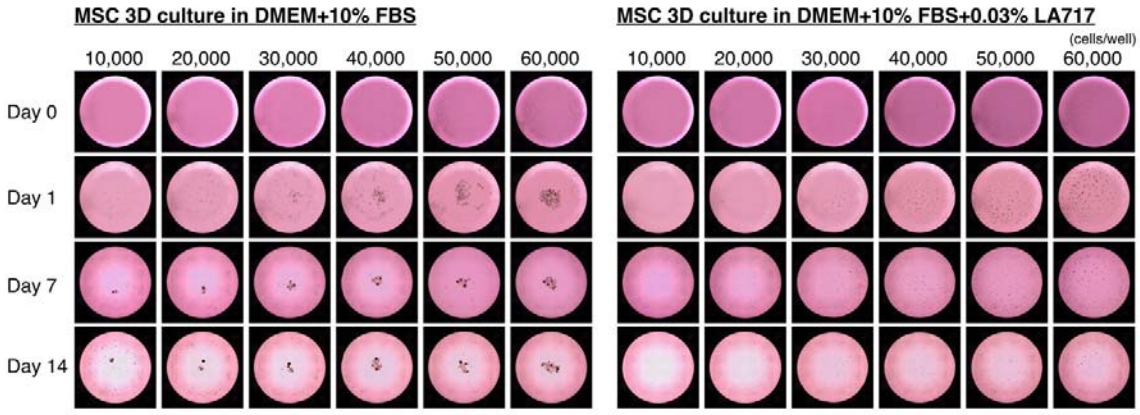

Figure 1. LA717 prevents cell aggregation in high-density three-dimensional (3D) cultures of human mesenchymal stem/stromal cells (MSCs). Representative whole-well images showing the time course of MSC cultures over 14 days in low-adhesion 96-well plates under two different 3D culture conditions. MSCs were seeded at densities ranging from 10,000 to 60,000 cells/well in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; left) or DMEM supplemented with 10% FBS and 0.03% LA717 (right; developed by Nissan Chemical Corporation, Tokyo, Japan). In the absence of LA717, MSCs aggregated rapidly, particularly at higher seeding densities, and formed dense clusters by day 1 that persisted throughout the culture period. In contrast, the addition of 0.03% LA717 effectively suppressed cell aggregation, maintaining a dispersed distribution of MSCs across all densities over the entire 14-day period. All images were acquired using the Cell3iMager duos2 (SCREEN Holdings). The inner diameter of each well is 6.4 mm.

Although slight aggregation was observed at higher cell densities (50,000 and 60,000 cells/well), these aggregates were transient and shrank over time, indicating that LA717 effectively promoted a stable dispersed state of MSCs under high-density conditions.

Culture conditions to optimize high-density 3D MSC culture

In this study, we investigated the effects of LA717 concentration and medium volume on the aggregation of MSCs in cultures in 3D using high-density cultures of 60,000 cells/well. MSCs cultured without LA717 showed significant aggregation regardless of the medium volume. However, the addition of LA717 effectively suppressed aggregation in a concentration-dependent manner, with high concentrations of LA717 (0.04%–0.06%) showing more pronounced effects (Figure 2). Furthermore, large medium volumes (300–400 μL) were associated with a greater decrease in MSC aggregation, even at low LA717 concentrations (0.03%–0.04%). These results suggest that both the LA717 concentration and medium volume play crucial roles in preventing MSC aggregation in high-density 3D culture.

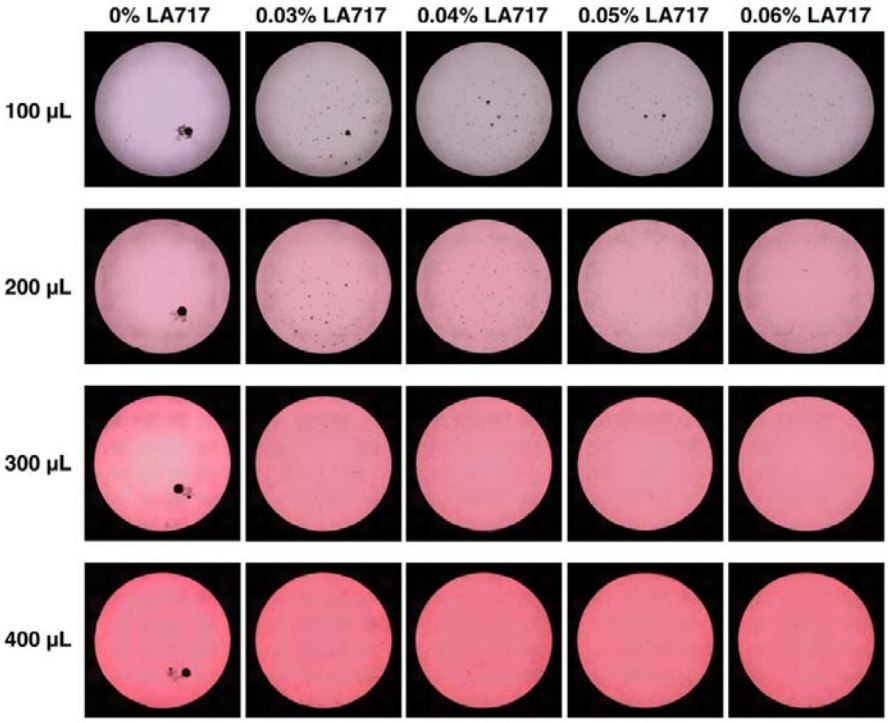

Figure 2. LA717 suppresses MSC aggregation in a concentration- and volume-dependent manner in 3D culture. Representative whole-well images showing the effects of LA717 concentration and culture medium volume on MSC aggregation after 14 days of 3D culture. MSCs (60,000 cells/well) were cultured in low-adhesion 96-well plates with Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) containing varying concentrations of LA717 (0%–0.06%) and medium volumes (100, 200, 300, and 400 µL). In the absence of LA717, MSCs aggregated extensively regardless of the medium volume. The addition of LA717 progressively suppressed aggregation in a concentration-dependent manner, with higher concentrations (≥0.04%) and greater medium volumes (≥300 µL) showing improved dispersion. These findings suggested that both LA717 concentration and medium volume are important

parameters for achieving a stable, high-density 3D culture of MSCs. All images were acquired using the Cell3iMager duos2 (Screen Holdings). The inner diameter of each well is 6.4 mm.

A single HeLa cell co-cultured with MSCs in an LA717-supplemented medium proliferated in an anchorage-independent manner and formed a colony

Next, we examined the behavior of MSCs and a single HeLa-GFP cell co-cultured in a 3D environment with medium supplemented with 0.03% LA717 (200 µL per well). All cells were prelabeled with the CVC dye for fluorescence imaging (Figure 3).

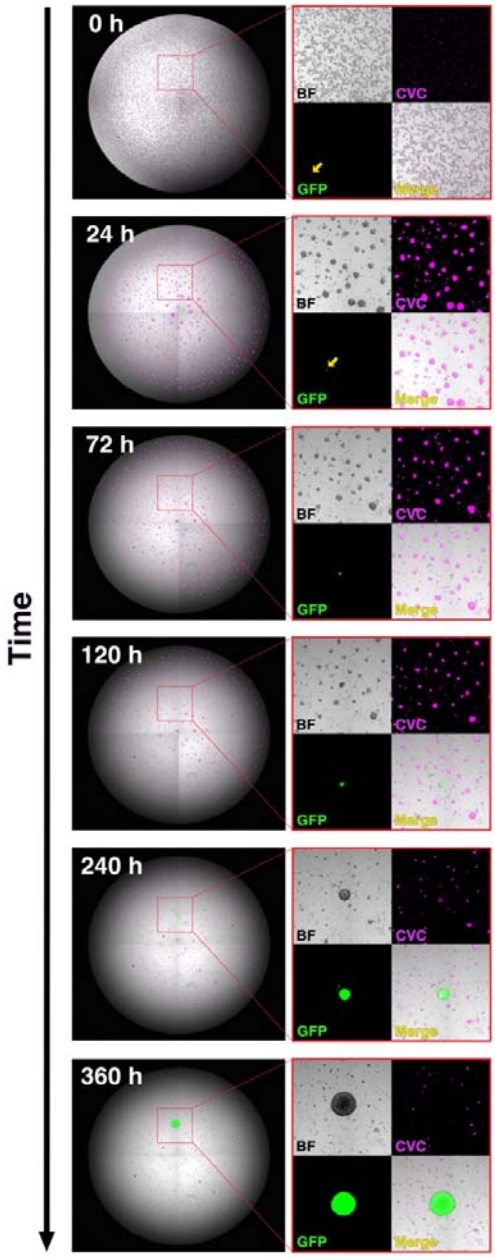

Figure 3. Transformed HeLa-green fluorescent protein (GFP) cells exhibit anchorage-independent proliferation and colony formation in a 3D culture environment with 0.03% LA717, unlike surrounding MSCs. Representative images extracted from time-lapse imaging performed every hour for

360 h (15 days), showing the behavior of a single HeLa-GFP cell co-cultured with 20,000 MSCs in 200 μL of LA717 (0.03%)-supplemented medium in a low-adhesion 96-well plate. All cells were prelabeled with CellVue Claret (CVC; pseudo-colored magenta) for fluorescence visualization. Bright-field (BF), GFP, CVC, and merged images at six-time points revealed that, while MSCs remained dispersed and did not proliferate, the single HeLa-GFP cell consistently proliferated in an anchorage-independent manner, ultimately forming a distinct colony. The GFP signal expanded over time, highlighting the dynamic growth of the transformed cells. A stitched movie at all the time points is provided in the Supplementary Movie. Images were acquired using a CellVoyager CQ1 confocal imaging cytometer (Yokogawa Electric). The inner diameter of each well is 6.4 mm.

Bright-field and CVC fluorescence (pseudo-colored in magenta) images in Figure 3 demonstrated that MSCs remained dispersed and showed no anchorage-independent growth under this 3D culture condition. Minor initial aggregation of MSCs was observed, which resolved over time. In contrast, the single HeLa-GFP cell exhibited anchorage-independent growth, doubled within 24 h, and continued to proliferate, ultimately forming a distinct colony. GFP fluorescence highlighted the dynamic HeLa cell colony formation process over 15 days. However, the fluorescence of the CVC dye in colonies derived from HeLa-GFP cells almost disappeared, likely due to the

continuous dilution of the fluorescent label as the cells divided. These results suggest that the 3D culture system utilizing LA717 effectively distinguishes the proliferative capacity of abnormally transformed cells, such as HeLa cells, from that of normal MSCs. The images in Figure 3 show a section at each time point when time-lapse imaging (360 h, imaging at 1 h intervals) was performed, and the video showing all the images for each time point stitched together is shown in the Supplementary Movie.

Single HeLa cells consistently formed colonies with a steady efficiency across different 3D culture conditions in the presence of LA717

We previously established a method for calculating the CFE of reference transformed cells, which serves as an indicator of the detection performance of transformed cells in the D-SACF assay (an approach that allows the efficient analysis of a large number of cells). Conventionally, the CFE is determined based on the total number of counted colonies obtained from multi-well image analysis. However, in this study, we developed and implemented a new approach whereby the CFE is calculated based on the total number of wells containing at least one colony rather than the number of counted colonies. Using this method, we assessed the CFE of HeLa-GFP cells under various culture conditions.

First, we evaluated the CFE of HeLa-GFP cells co-cultured with MSCs in a medium containing LA717 at varying concentrations (0.03%–0.06%). We seeded cells into a total of 240 wells (60 wells × 4 plates) under conditions where each well had 200 μL of medium with 20,000 MSCs and an average of 0.5 HeLa-GFP cells (i.e., $\lambda = 0.5$ cells/well). The cells were then co-cultured in a 3D environment, and colony analysis was performed after 3 weeks. The results of three independent runs of the CFE of HeLa-GFP cells at four different concentrations of LA717 (0.03%, 0.04%, 0.05%, and 0.06%) are shown in Figure 4a. The data were analyzed using one-way repeated measures ANOVA, which revealed no significant effects of LA717 concentration on the CFE of HeLa-GFP cells. These findings indicate that the CFE of HeLa-GFP cells remained stable regardless of the LA717 concentration. This stability suggests that LA717 did not influence the anchorage-independent growth capacity of HeLa-GFP cells within the tested concentration range.

Next, to evaluate the impact of seeding conditions on the CFE of HeLa-GFP cells, we conducted three independent runs under fixed culture conditions: 200 μL of medium containing 0.03% LA717 and 20,000 MSCs per well. We varied the seeding ratio of HeLa-GFP cells ($\lambda = 0$–1.0 cells/well) and the total number of seeded wells (60–840 wells). After 3 weeks of culture, CFE was analyzed for each condition. The results

showed that the CFE of HeLa-GFP cells remained consistent across all tested seeding conditions (Figure 4b). Statistical analysis using one-way repeated measures ANOVA revealed no significant effect of the HeLa-GFP seeding ratio on CFE. These findings indicate that under the examined conditions, CFE evaluation of reference transformed cells is robust and reliable. Importantly, in the control condition (λ = 0; MSCs only, 60 wells), no colony formation was observed, confirming the specificity of colony detection for HeLa-GFP cells.

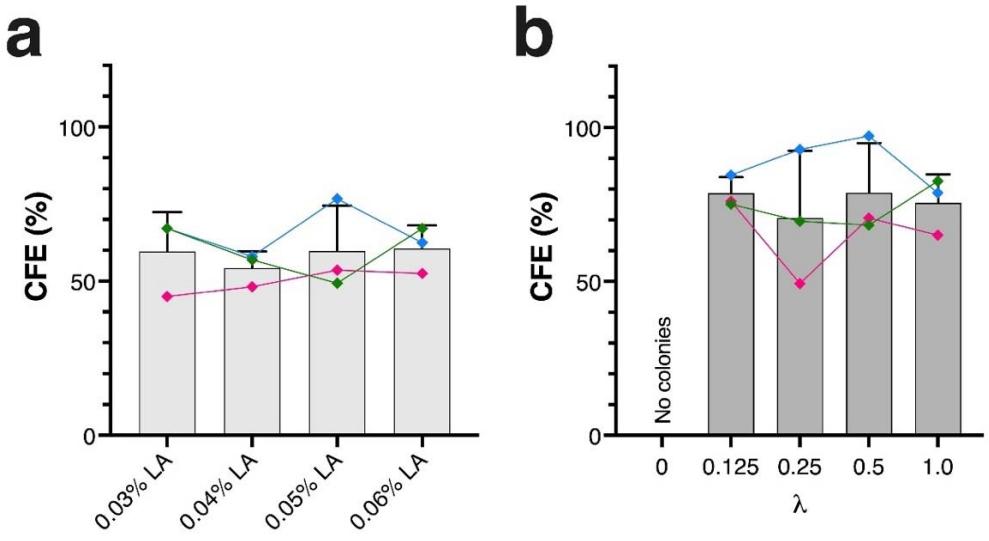

Figure 4. The colony-forming efficiency (CFE) of HeLa-GFP cells co-cultured with MSCs in LA717-supplemented medium under various culture conditions. (a) The CFE of HeLa-GFP cells was evaluated under four different concentrations of LA717 (0.03%, 0.04%, 0.05%, and 0.06%) in 200 μL of

culture medium per well. HeLa-GFP cells (0.5 cells/well on average) were co-cultured with 20,000 MSCs per well in a 96-well plate (*n* = 240 wells per condition) for 3 weeks. CFE was calculated based on the number of wells containing colonies derived from HeLa-GFP cells, as determined by image analysis. Bar graphs represent the mean and SD values from three independent experiments, and the colored lines show the individual results from each experiment. **(b)** The CFE of HeLa-GFP cells was evaluated under varying cell input amounts (λ = 0.125, 0.25, 0.5, and 1.0) in 200 µL of medium containing 0.03% LA717 and 20,000 MSCs per well. HeLa-GFP cells were seeded into 840, 420, 240, and 120 wells, respectively, and cultured for 3 weeks. The CFE was calculated from the number of wells containing colonies detected by image analysis. Bars represent the mean and SD values of three independent experiments, and colored lines indicate the individual results. No colony formation was observed under the control condition (λ = 0; i.e., MSCs only, 60 wells), confirming the specificity of HeLa-GFP-derived colony detection. Detailed numerical data for each condition in **(a)** and **(b)** are provided in Supplementary Tables 1 and 2, respectively. The equation used to calculate CFE in **(a)** and **(b)** is described in Supplementary Figures 1 and 2, respectively. LA, LA717.

Supplementary Tables 1 and 2 present the CFE of the reference transformed cells under each culture condition, calculated using two distinct methods: (1) based on the total number of wells containing at least one colony, as applied in this study, and (2) based

on the conventional method of counting the total number of colonies. The results from both methods were consistent, indicating that the CFE of the reference transformed cells remained nearly identical regardless of the calculation method employed.

Improved visualization and detection of colonies using fluorescence staining in LA717-based 3D culture

To enhance the efficiency and accuracy of colony detection through the image analysis of whole-well images in a 96-well plate, we explored the application of fluorescence staining in cultured samples. Based on our previous studies, we evaluated nuclear, mitochondrial, and lysosomal fluorescent labeling for this purpose. Figure 5a shows representative images of a cell colony observed after co-culturing 0.5 HeLa-GFP cells with 20,000 MSCs per well in a medium supplemented with 0.03% LA717 for 3 weeks. The presence of GFP fluorescence in the cell aggregates identified in the bright-field images confirmed that these colonies originated from HeLa-GFP cells. The addition of fluorescent dyes specific to the nucleus, mitochondria, and lysosomes during cell culture enabled the organelle staining of live cells, leading to clear fluorescent labeling of HeLa-GFP-derived colonies. Among the dyes used, LysoTracker and MitoTracker are selective for live cells, which indicated that the colonies consisted of viable cells.

Figure 5b shows colonies formed by HeLa-WT cells under the same culture conditions. Live staining with Calcein-AM resulted in strong fluorescence signals, confirming that the colonies were composed of viable cells. Based on the findings from HeLa-GFP cells, the colonies observed under these conditions were likely derived from HeLa-WT cells.

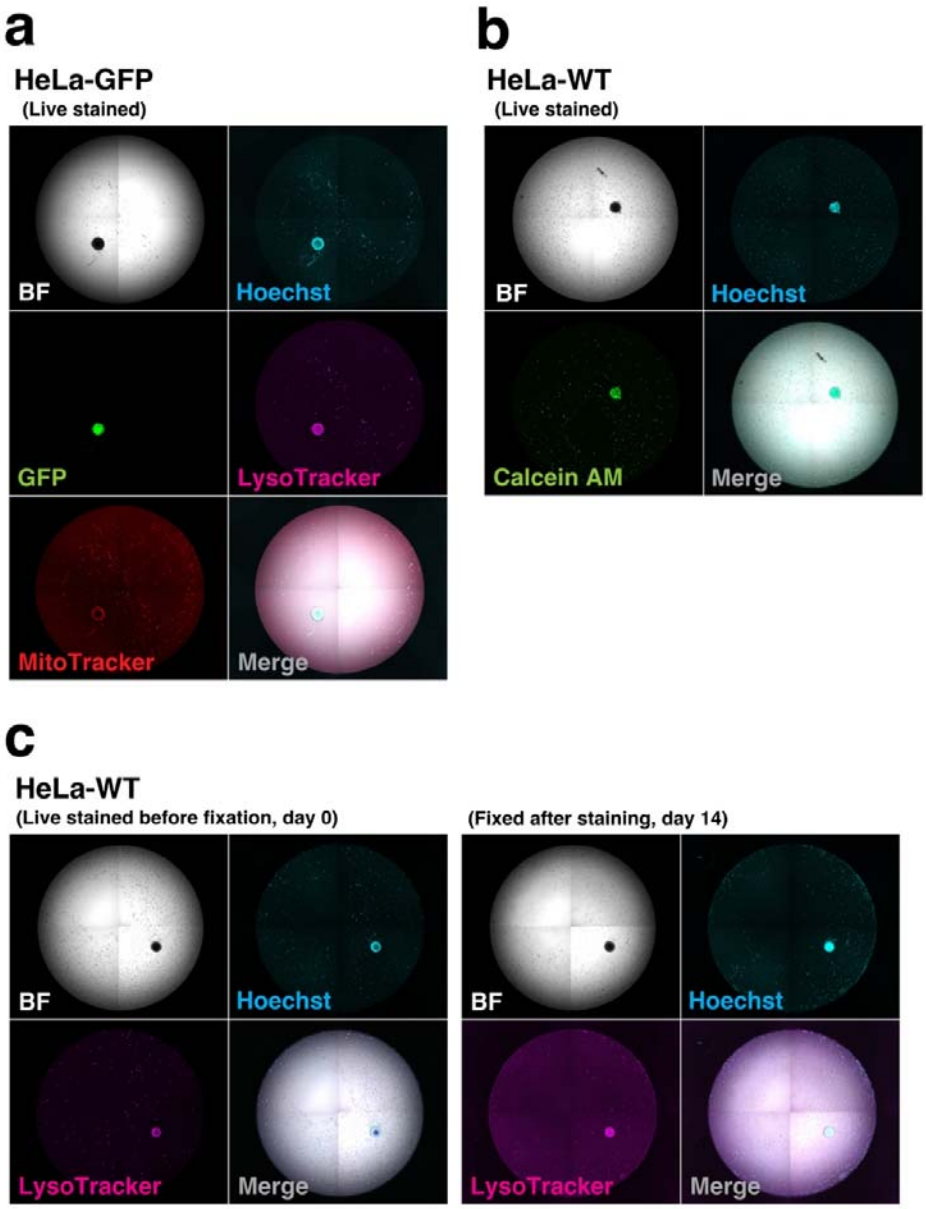

Figure 5. Fluorescence-based detection of colonies formed by single HeLa cells co-cultured with MSCs in LA717-supplemented medium. Representative images of colonies formed by a single HeLa-GFP or wild-type HeLa cell co-cultured with 20,000 MSCs per well for 3 weeks in 0.03% LA717-supplemented medium (200 µL/well). Fluorescent staining markedly enhanced the accuracy of colony detection in image analysis. Images were acquired using the CellVoyager CQ1 confocal imaging cytometer (Yokogawa Electric). **(a)** A colony derived from a single HeLa-GFP cell. Live staining was performed by adding Hoechst 33342 (nucleus), MitoTracker Deep Red (mitochondria), or LysoTracker Red (lysosomes) to the culture medium and incubating the cells for a defined period. The GFP signal confirmed that the colony originated from HeLa-GFP cells, which was also clearly visualized with each dye. **(b)** A colony derived from a single wild-type HeLa cell. Live staining with Hoechst 33342 and Calcein-AM revealed strong fluorescence signals, indicating colony viability. **(c)** (Left) A wild-type HeLa colony stained in the live state with Hoechst 33342 and LysoTracker Red. (Right) The same colony was imaged 14 days after fixation with 1% paraformaldehyde (PFA). Fluorescence signals from Hoechst and LysoTracker staining were retained, indicating that this dye combination is compatible with fixation and long-term storage, allowing flexible scheduling of image acquisition and analysis. The inner diameter of each well is 6.4 mm.

Fluorescent staining has proven useful for image-based colony detection. To enhance its applicability, we further investigated fixation methods for stained cells. As shown in Figure 5c, careful temperature management during fixation allowed fluorescence signals from LysoTracker and Hoechst staining (left images) to be preserved for at least 14 days (right images show LysoTracker and Hoechst staining). This approach increases the flexibility of the analytical process by enabling imaging-based colony evaluation over an extended period, thereby improving the practicality of the method.

These fluorescent labeling techniques enabled clear visualization of colonies derived from transformed cells in the LA717-based 3D culture environment. Consequently, similar to the previous D-SACF assay, detecting transformed cell-derived colonies and evaluating the CFE of reference cells through image analysis became readily achievable.

Discussion

In a previous study conducted by the developers of LA717 [11], it was shown that culturing cells in low-attachment vessels with LA717-supplemented medium results in a uniform dispersion of cells throughout the medium, preventing adhesion and aggregation among neighboring cells while preserving contact between newly divided

cells and minimizing medium convection. These properties also keep cells and spheroids localized and ultimately led to the proposal of a novel 3D culture system utilizing these unique characteristics. Building on these findings, we demonstrated that MSCs, which readily aggregate under high-density conditions, remained stably dispersed in theLA717-containing medium without proliferating, with each cell maintaining its position. These features suggest that LA717 is particularly advantageous for scalable and uniform MSC culture, as it reduces aggregation-related issues. In contrast, tumorigenic HeLa cells exhibited anchorage-independent growth from single cells and consistently formed colonies under the same culture conditions. Furthermore, through fluorescent labeling, we successfully enhanced the visualization of colonies derived from transformed cells within the LA717-based 3D culture environment, enabling more accurate image-based colony detection and simplified quantitative evaluation of the CFE of reference cell lines such as HeLa. Collectively, these findings support the utility of the LACF assay—based on a 3D culture system using a non-viscous, LA717-supplemented liquid medium that does not require stringent temperature control for preparation or handling —as a more practical alternative to conventional soft agar-based methods for assessing malignant transformation in vitro. Finally, we established and systematized the D-LACF assay as a digital evaluation

platform capable of estimating the proportion of transformed cells in hCTPs by detecting colony formation at the single-cell level (Figure 6). By revising the assay workflow from our previous study [10], this approach updates the entire testing pipeline—from assay performance evaluation to product testing and data interpretation—offering greater feasibility and scalability than the original D-SACF assay and paving the way for large-scale screening of transformed cells in hCTPs.

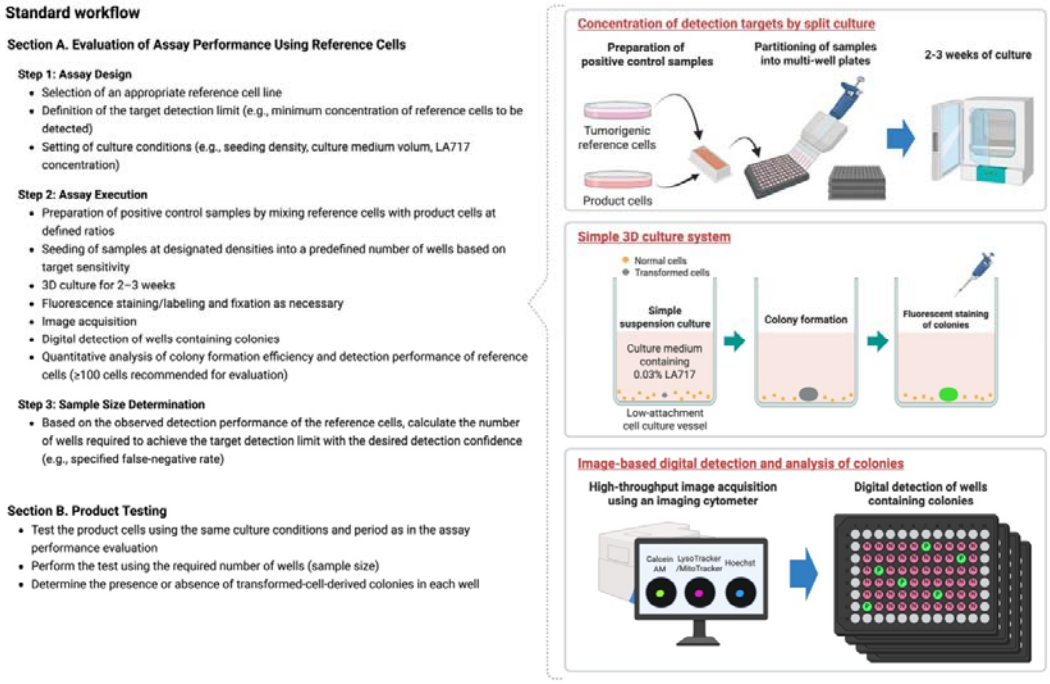

Figure 6. Schematic overview of the digital liquid/low-molecular-weight agar colony formation (D-LACF) assay. The D-LACF assay detects transformed cells by combining LA717-based 3D culture with image□based digital analysis. Section A illustrates the evaluation of assay performance using a reference cell line: (1) assay design, (2) assay execution—split culture, 3D growth in

LA717-supplemented medium, fluorescent labeling, and high-throughput colony detection—and (3) determination of sample size based on the observed detection performance. Section B presents product testing conducted under the same culture conditions but with the predetermined sample size (number of wells) calculated in Section A; each well is then classified as positive (P) or negative (N) for transformed-cell-derived colonies. This figure was created with BioRender.com.

Another *in vitro* 3D culture system designed to evaluate cellular transformation is the growth in low-attachment assay (GILA), which uses only standard cell culture medium and low-adhesion culture vessels to easily assess anchorage-independent growth [15,16]. A recent report showed that GILA can detect cell transformations resulting from specific gene mutations with a sensitivity comparable to that of the conventional SACF assay [17]. The possibility of multiple assay options is advantageous as it enables the selection of the most appropriate method based on various requirements. The standard GILA protocol recommends seeding several hundred to several thousand cells per well in a 96-well plate. However, in this study, when MSCs were seeded at 10,000 cells/well in a conventional culture medium, pronounced cell aggregation was observed. In contrast, the LA717-supplemented medium effectively suppressed MSC aggregation under the same seeding conditions. These results suggest that for quality assessment of hCTPs composed of a large number of cells—particularly for detecting a trace amount

of transformed cells—GILA may not fit the purpose due to its limitations under high-density culture conditions. Instead, the LACF assay, which enables high-density culture by modifying the culture medium composition, is currently the most practical and effective method for such applications.

Although the fundamental principle of D-SACF/D-LACF assay is similar to that of droplet digital PCR (ddPCR), its technical concept can be divided into three main components: (1) Fractionating the test cells into multi-well plates for 3D culture, thereby enriching the target transformed cells and improving the signal-to-noise ratio for image analysis; (2) Utilizing high-throughput imaging equipment to efficiently analyze multi-well images; and (3) Performing digital detection (presence/absence) of colonies in each well, enabling the estimation of the proportion of transformed cells. However, a critical difference exists between ddPCR and D-SACF/D-LACF assay concerning point number (3). In ddPCR, when a target sequence is present within a partition, it is theoretically amplified with nearly 100% efficiency, rendering the partition positive [18]. In contrast, in the D-SACF/D-LACF assay, even if a transformed cell is present in each partition, not every cell will necessarily form a colony. Hence, it is challenging to infer the proportion of transformed cells based solely on the digital detection of colonies. This is a fundamental premise for any assay designed to detect

transformed cells in which the characteristics of potentially transformed cells in the product are inherently unknown. Therefore, in the D-SACF/D-LACF assay, a known transformed cell line, such as HeLa, was designated as the reference and positive control, and its estimated CFE (i.e., detection rate) was determined in advance as an indicator of the performance of the assay. If no colonies derived from the transformed cells were detected upon testing the product cells, it was interpreted that the proportion of transformed cells equivalent to the reference cells in the product fell below the estimated threshold (i.e., detection limit). This interpretation is defined as a product's quality attributes. This constitutes the fundamental workflow of this assay [10].

In this study, while systematically establishing the D-LACF assay, we updated a portion of the performance evaluation process that was originally conducted in the prior D-SACF assay. When evaluating the CFE of reference cells, an important performance indicator, we focused on the following two key operational issues: the complexity of preparing low-concentration cell suspensions and the high susceptibility of CFE to variability during the cell spiking process. Therefore, to enable a more accurate and consistent CFE assessment, we optimized the protocol for these critical steps. First, we refined the procedure for counting low-concentration cell suspensions (approximately 100 cells/mL). Instead of dispensing 10 μL into multiple wells of a Terasaki plate and

manually counting cells under a microscope, we dispensed the same volume into a 384-well plate, performed fluorescent staining, and used imaging-based methods for cell counting. This approach partially automates the cell counting process, thereby streamlining the workflow while potentially increasing accuracy. In addition, we considered the Poisson distribution and its 95% CI when spiking the low-concentration reference cell suspensions. Because cell seeding at such low concentrations is prone to sampling errors, taking this statistical variability into account allowed us to define a confidence range in the performance metrics, which is expected to improve the analytical accuracy of product testing. Furthermore, we introduce a novel method to calculate the CFE of the reference cells. Without time-lapse tracking, it is difficult to determine exactly how many seeded cells give rise to the total number of colonies in a single well after a specified culture period. Therefore, we calculated the CFE by taking the ratio of the estimated number of seeded cells to the total colony count. However, accurately counting the colonies in each well can be challenging because of the complexities of image analysis, particularly when multiple colonies overlap or come into contact with each other. To address this issue, we devised a new calculation method using a simple formula that based the CFE on the number of wells containing at least one colony. In this approach, it is only necessary to determine whether a colony is

present in each well, which eliminates the need for detailed colony counting and greatly simplifies the image analysis process. The CFE values obtained using both the conventional and newly proposed methods were largely consistent and remained relatively stable, regardless of culture conditions such as variations in LA717 concentration or the seeding density of HeLa-GFP cells. These results highlight the robustness and reliability of the proposed method for evaluating the CFE of reference cells as part of the D-LACF assay's performance assessment. Given that the new approach offers both procedural simplicity and the potential for enhanced accuracy and reproducibility, we have adopted it as the standard method for future applications.

These findings suggest that, similar to the D-SACF assay, the D-LACF assay can detect a single HeLa cell mixed with a large number of MSCs in each well of a multi-well plate with a certain degree of precision. According to previous studies [11], it is theoretically possible for the D-LACF assay to detect HeLa cells at a level of 0.00001% in MSCs (i.e., one HeLa cell per 10 million MSCs); however, further detailed verification of the detection sensitivity and limits of D-LACF is necessary. Moreover, although previous reports have suggested that multiple cell lines can proliferate in a 3D culture environment supplemented with LA717, the CFEs of these cell lines have not been thoroughly investigated [11]. Our preliminary findings indicate some potential in

this regard; however, determining which cell lines can serve as reference cells for the D-LACF assay and verifying its applicability to cell types other than MSCs remain ongoing challenges. Future studies should explore the versatility and scope of the D-LACF assay.

Because the performance of the D-LACF assay depends heavily on the accuracy of the image analysis, there remain areas that can be improved. For example, in conventional soft agar cultures, aggregated normal cells may be misidentified as colonies during image analysis, resulting in false-positive results. Similarly, in a 3D culture system supplemented with LA717, insufficient mixing of the cell suspension can lead to the aggregation of normal cells, making it difficult to distinguish them from true colonies. Therefore, we employed a method in which the cell membranes of the test cells were prelabeled with CVC. This approach enabled a clear distinction between simple cell aggregates and colonies formed through cell proliferation. Specifically, cell aggregates initially exhibited strong fluorescence but typically did not proliferate and gradually diminished over time. In contrast, colonies formed by proliferating cells showed a gradual decrease in fluorescence intensity owing to successive cell divisions. These observations suggest that incorporating parameters such as the total and average fluorescence intensities of objects into the image analysis process could facilitate an

accurate distinction between proliferative colonies and non-proliferative cell aggregates. However, when Hoechst 33342, LysoTracker, or MitoTracker is used, debris may be non-specifically stained, potentially resulting in false-positive colony determination. Currently, it is difficult to make a final decision regarding the presence of colonies in each well solely through image analysis, and in some cases, manual inspection of bright-field images is necessary. In the future, developing and introducing fluorescent dyes with high specificity for live or highly proliferative cells, as well as exploring analysis methods for label-free images that incorporate AI technologies such as machine learning or deep learning, are expected to enhance the accuracy of colony detection.

In product cell testing, the ideal outcome is that no colonies are detected. However, if colony-like structures are observed and their status as true or false positives is uncertain, the LACF assay offers a complementary option that the conventional SACF assay lacks. Because the LA717-based 3D culture system uses a liquid medium, suspect cell aggregates can be easily isolated and retrieved with a micropipette. The collected cells can then be sub-cultured to determine whether they proliferate, allowing for further characterization to establish whether they are truly malignant. Moreover, a detailed analysis of any unexpectedly tumorigenic cells may provide new opportunities to investigate the mechanisms of cellular transformation and develop strategies for its

suppression, thereby widening the research applications of this novel 3D culture platform.

The workflow established in this study for the D-LACF assay has the potential to serve as a basis for test design for the quality assessment of various hCTPs. A key feature of this approach is its flexibility and versatility, which allow for the adjustment of test conditions (e.g., cell seeding density, number of seeding wells, or LA717 concentration) based on the specific properties of each product and the construction of an optimal test design. Furthermore, the D-LACF assay is expected to improve the quality and safety of hCTPs, ultimately leading to the development of more reliable products and advancements in therapies that utilize them. To promote the broader adoption of the D-LACF assay, we plan to further optimize the basic protocol and conduct analytical validation of the assay performance through multisite studies. We will actively share and disseminate the results of such validations, highlighting the versatility and scope of the assay, with the ultimate aim of standardizing the D-LACF assay internationally.

Conclusion

In this study, we successfully established the D-LACF assay as a practical and sensitive platform for detecting trace amounts of malignantly transformed cells in hCTPs. By

combining a novel LA717-based 3D culture system with digital image analysis and refined evaluation protocols, the assay offers improved feasibility, accuracy, and scalability compared to conventional methods. These findings provide a foundation for the broader implementation of the D-LACF assay in quality testing frameworks and contribute to enhancing the safety and reliability of future hCTPs. Moving forward, continued optimization and inter-laboratory validation of this assay will be essential for its practical implementation and international standardization as a quality control tool for advanced cell-based therapies.

Declaration of Competing of Interest

The authors have no commercial, proprietary, or financial interest in the products, methodologies, or companies described in this article.

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Author Contributions

Study conception and design: SK and SY. Data acquisition: SK and TY. Analysis and interpretation of data: All authors. Manuscript drafting and revision: SK and SY. All the authors have approved the final manuscript.

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Supplementary materials

Supplementary figures and tables associated with this article are included in the "Supplementary Material" file attached separately.

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