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Detailed method description for noninvasive monitoring of differentiation status of human embryonic stem cells

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ABSTRACT

The (non)differentiation status of human embryonic stem cells (hESCs) is usually analyzed by determination of key pluripotency defining markers (e.g., OCT4, Nanog, SOX2) by means of reverse transcription quantitative polymerase chain reaction (RT–qPCR), flow cytometry (FC), and immunostaining. Despite proven usefulness of these techniques, their destructive nature makes it impossible to follow up on the same hESC colonies for several days, leading to a loss of information. In 2003, an OCT4–eGFP knock-in hESC line to monitor OCT4 expression was developed and commercialized. However, to the best of our knowledge, the use of fluorescence microscopy (FM) for monitoring the OCT4–eGFP expression of these cells without sacrificing them has not been described to date. Here, we describe such a method in detail, emphasizing both its resolving power and its complementary nature to FC as well as the potential pitfalls in standardizing the output of the FM measurements. The potential of the method is demonstrated by comparison of hESCs cultured in several conditions, both feeder free (vitronectin, VN) and grown on feeder cells (mouse embryonic fibroblasts, MEFs).
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Human embryonic stem cells (hESCs)[2] are characterized by their unlimited proliferation potential (self-renewal) and their ability to differentiate into all cell types of the mesoderm, ectoderm, and endoderm germ layers (pluripotency). These hESCs, therefore, could potentially be useful in the field of regenerative medicine [1,2]. The process of differentiation has been explored extensively, but finding ways of keeping hESCs undifferentiated is equally essential for fundamental clinical research and toxicological screenings [3]. Commonly used markers for identifying this undifferentiated status include the two key pluripotent transcription factors OCT4 and Nanog [4,5].

Currently, the differentiation status is routinely measured using immunostaining, reverse transcription quantitative polymerase chain reaction (RT–qPCR), and/or flow cytometry (FC). Although these techniques have proven their usefulness in analyzing hESCs, it is necessary to sacrifice cells, making it impossible to monitor the same cells during the experiment. Here, we evaluated the applicability of a commercially available OCT4–eGFP knock-in hESC line (WiCell Research Institute, Madison, WI, USA) in combination with fluorescence microscopy (FM) for noninvasive examination of (non)differentiation of hESCs. This OCT4–eGFP knock-in hESC line (=OCT4 reporter hESC line) was developed in 2003 by means of homologous recombination whereby the transcription of enhanced green fluorescent protein (eGFP) is regulated by the promoter region of OCT4 [6]. The pluripotent status of hESCs can be verified in this hESC line by means of eGFP detection; a decrease in eGFP represents a decrease in OCT4 expression and, thus, a decrease in pluripotency, indicating that the hESC line is

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[2] *Abbreviations used:* hESC, human embryonic stem cell; RT–qPCR, reverse transcription quantitative polymerase chain reaction; FC, flow cytometry; FM, fluorescence microscopy; eGFP, enhanced green fluorescent protein; S/N ratio, signal-to-noise ratio; RA, retinoic acid; bFGF, basic fibroblast growth factor; MEF, mouse embryonic fibroblast; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; KO–SR, knock-out–serum replacement; NEAA, nonessential amino acid; DPBS, Dulbecco's phosphate-buffered saline; E8, Essential 8; ITS-A, insulin–transferrin–sodium selenite–sodium pyruvate supplement; UCM, unconditioned medium; PBS, phosphate-buffered saline; FS/SS, forward scattering/side scattering; SD, standard deviation.

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differentiating. This hESC line has been used for different purposes, for example, to analyze cell division and to create induced pluripotent stem cells [7,8]. Noninvasive monitoring of OCT4 can be advantageous to, for example, investigate the effect of different culture conditions on hESC pluripotency. To our knowledge, however, no detailed description of a methodology in which this hESC line is used in a nondestructive time lapse experiment has been published to date.

A nondestructive and fast way to define the differentiation status of the cells of this hESC line is to measure the fluorescence of the hESC colonies by means of FM. By determination of the densitometric means of a specific colony and the background, the signal-to-noise ratio (S/N ratio) can be compared between different conditions on a daily basis without any loss of cells. FC, on the other hand, allows determining fluorescence at the single-cell level and is arguably the "gold standard" despite its destructive nature. Our goal was to correlate the measurements of both techniques.

To validate this nondestructive method, hESCs were differentiated using 2 μM retinoic acid (RA). Their S/N ratio was compared with hESCs cultured in medium containing basic fibroblast growth factor (bFGF), a well-known growth factor to maintain self-renewal and pluripotency. Subsequently, feeder-free culture and feeder cell culture of hESCs were analyzed in parallel to determine the effect of mouse embryonic fibroblasts (MEFs) on our hESC colonies. FM measurements added valuable information in interpreting FC experiments. More specifically, the use of FM has the additional advantage that it allows monitoring of hESC colony morphology and colony homogeneity, which we demonstrate to be a considerable source of variance undetected at the single-cell level.

Finally, one application of this method is given where MEF conditioned medium (CM) is used to analyze its beneficial impact on hESC growth.

Materials and methods

Materials

All products were purchased from Life Technologies (Carlsbad, CA, USA) unless stated otherwise.

hESC culture on feeder cells

hESCs were cultured on feeder layers of inactivated MEFs. MEFs were grown to confluence in a T75 culture flask (37 °C, 5% CO_2, 5% O_2) using medium composed of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Inactivation of MEFs occurred by incubation with 10 μg/ml mitomycin C (Sigma–Aldrich, St. Louis, MO, USA) for 2.5 h at 37 °C. The cells were detached from a T75 flask with 0.25% trypsin–EDTA and plated on a precoated 0.1% gelatin 6-well plate at a density of 20,000 cells/cm^2 and cultured as described above. The next day, the WA01 Oct4–eGFP knock-in hESCs were plated on the MEFs and cultured in hESC medium consisting of DMEM/F12 with 20% knock-out–serum replacement (KO–SR), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids (NEAAs), 2 mM L-glutamine, and 4 ng/ml bFGF. Splitting of the cells was performed every 4 or 5 days with 0.5 mM EDTA in Dulbecco's phosphate-buffered saline (DPBS), based on the manufacturer's protocol for feeder-free splitting.

Differentiation of hESCs was induced by adding 2 μM RA and by removing bFGF from the hESC medium.

Table 1
Composition of different media used for feeder-free culture of hESCs.

Component	UCM−	UCM+	CM−	CM+
DMEM/F12	+	+	+	+
1.25% ITS-A	+	+	+	+
2.5 mM L-glutamine	+	+	+	+
1.25% NEAAs	+	+	+	+
MEF secretome	–	–	+	+
4 ng/ml bFGF	–	+	–	+

Feeder-free culture of hESCs

In addition to feeder cell culture, feeder-free conditions were also used to validate the method. For feeder-free culture, hESCs were plated on a precoated vitronectin (VN) plate (coating concentration = 0.5 μg/cm^2) and cultured in Essential 8 (E8) medium. Splitting was performed every 4 or 5 days with 0.5 mM EDTA in DPBS according to the manufacturer's protocol of culturing hESCs in Essential 8 medium. Differentiation of hESCs was induced by adding 2 μM RA and by removing bFGF from the hESC medium used in feeder cell culture.

For the application of the screening methodology, different media were tested. The compositions of these media are summarized in Table 1.

Insulin–transferrin–sodium selenite–sodium pyruvate supplement (ITS-A) CM was made by adding 15 ml of ITS-A unconditioned medium (UCM) to an inactivated MEF T75 flask (20,000 cells/cm^2). After culturing for 24 h, CM was collected and filtered through a 0.22-μm Sterivex GP filter unit (Millipore, Bedford, MA, USA) for removal of cells and cell debris.

Fluorescence microscopy and data analysis

After removal of medium, eGFP expression (Ex = 485 nm, Em = 515 nm, exposure time = 5000 ms) of 6 colonies/condition was measured daily on an Axiovert 200M inverted fluorescence microscope equipped with the Axiovision multichannel fluorescence module and an AxioCam MRM camera (Carl Zeiss, München, Germany). Colonies were screened at 10× magnification using a Carl Zeiss short distance Plan-Acromat objective (Carl Zeiss) and visualized using Zeiss filter set number 38 (BP 470/40, FT 495, BP 525/50). For larger colonies, different TIFF images were stitched using Photoshop CS4 (Adobe, San Jose, CA, USA). The S/N ratio was determined by dividing the densitometric mean of the colony by the densitometric mean of the background.

Flow cytometry

In general, FC was carried out at the end of each experiment (day 5). To obtain single cells, all cell cultures were incubated with 0.25% trypsin–EDTA for 6 min. Prior to FC analysis, cells were resuspended in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) solution. Flow count beads (Analis, Suarlée, Belgium) were added to acquire absolute cell counts. The different conditions were analyzed using Beckman Coulter Cytomics FC500 and CXP analysis software. A minimum of 10,000 events was acquired for each condition.

Celigo S

A Celigo S imaging cell cytometer (Brooks, Poway, CA, USA) was used to evaluate the confluency during the experiment (from day 0 to day 5). Confluency can be defined as the total coverage of the plate. Medium was removed because of autofluorescence, and

PBS was added to the culture to prevent dehydration during measurement due to the warmth generated by the system.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (San Diego, CA, USA). Differences were evaluated by a Student's *t* test. A *P* value <0.05 was considered as statistically significant.

Results

Method optimization of noninvasive monitoring of differentiation status of hESCs

During this method optimization, a detailed comparison was made between monitoring OCT4 expression by means of noninvasive FM and by "destructive" FC as the gold standard.

Assessing autofluorescence

Autofluorescence of cells due to the presence of cellular metabolites such as nicotinamide adenine dinucleotide hydride (NADH) is a well-known issue and must be investigated to avoid misinterpretation of fluorescence data [9]. The autofluorescence of MEFs was determined by FM and FC, and a nonreporter hESC line (UGENT2 [10]) was included as a negative control.

Fluorescence microscopy. For FM, S/N ratio is measured by dividing the densitometric mean of the colony by the densitometric mean of its background (Fig. 1A). For feeder cultures, therefore, it is essential to determine the background noise that might derive from the autofluorescence of the MEFs. The MEF signal is not detectable near hESC colonies and, as such, has no impact on the S/N ratio when compared with hESCs growing in feeder-free culture (on VN) (Fig. 1A).

Flow cytometry. Next, these FM measurements were compared with FC. The fluorescence histogram of the OCT4 reporter hESC line (cells detached from a VN plate), from MEFs, and of a nonreporter hESC line (UGENT2, cells detached from a VN plate), used as a baseline control, is presented in Fig. 1B. No autofluorescence was observed in the UGENT2 cell line (fluorescent signal < 10^0). MEF autofluorescence, however, is clearly present but is 10-fold less compared with the true signal of the OCT4 reporter hESC line.

Note that the autofluorescence from MEFs will have an influence on the fluorescence histogram of detached hESCs from a plate cultured on MEFs. Gating on the forward scattering/side scattering (FS/SS) plot to exclude the MEFs from the histogram is not possible because no distinction could be made between MEFs and hESCs in terms of FS/SS. However, the relative portion of the inactivated MEFs compared with the growing colonies decreases over time. Because FC is used only at the end of the subsequent experiments, only a small contribution of the MEFs (<10%) to the fluorescence histogram is expected for feeder cell experiments.

Yet, even with decreasing signal during differentiation, caution needs to be taken when directly comparing the values of FC measurements of hESCs grown on MEFs and on VN.

To assess the resolving power of the FC, complete differentiation of the OCT4 reporter hESC line (by means of 2 μM RA, culture on VN) was performed. The fluorescent signal rapidly decreased during the first 6 days and completely disappeared after 15 days in culture, falling back to the same level as the UGENT2 line, with no detectable autofluorescence (data not shown). Further experiments in this study were done on 6-day cultures because these showed adequate reduction in fluorescent signal.

Fluorescent signal in OCT4–eGFP knock-in hESC line on MEFs and VN during differentiation

Fluorescence microscopy. The resolving power of the FM to determine differences in differentiation status was verified as described below. The OCT4 reporter hESC line was cultured on feeder cells (MEFs) in three different media to investigate non-differentiation (hESC medium with 4 ng/ml bFGF), spontaneous differentiation (hESC medium without bFGF), and directed differentiation (hESC medium with 2 μM RA), respectively, and was also compared with feeder-free conditions (human recombinant VN in two different media (E8 medium and hESC medium with 2 μM RA). Only the conditions with media that contain bFGF are assumed to keep the hESC undifferentiated [11]. Colony fluorescence (5/6 colonies per condition) was assessed daily for 6 days, and results were

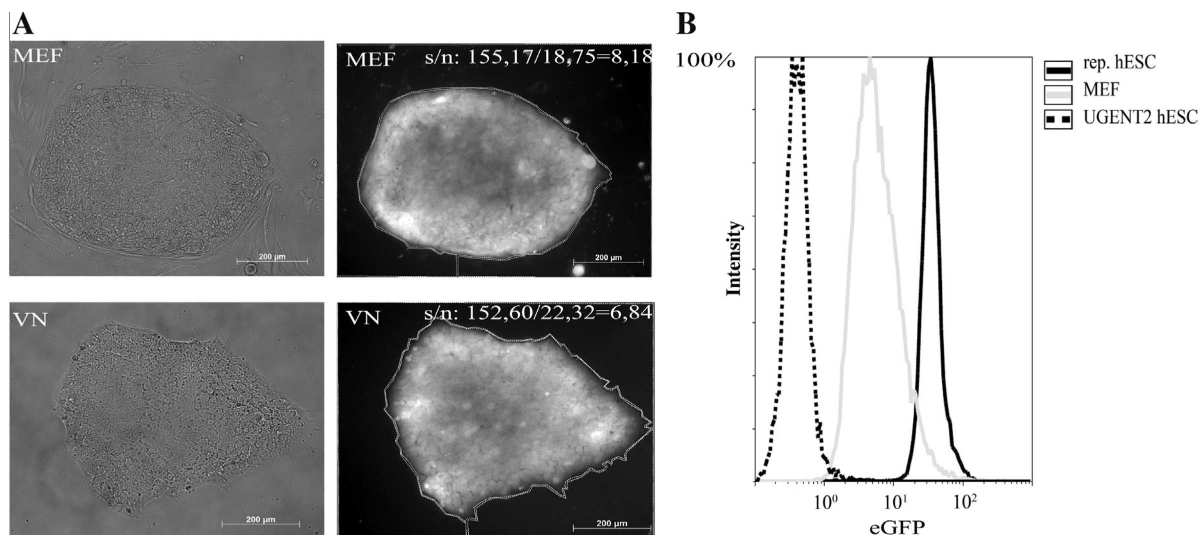

Fig.1. Autofluorescence of MEFs and hESCs. (A) Brightfield image (left) and fluorescence image (right) of an OCT4 reporter hESC colony cultured on MEFs (top) and on VN (bottom) obtained by FM. No autofluorescence of the MEFs can be observed. The dotted white lines in the right panels illustrate how colonies are manually delineated in all experiments to assess the S/N ratio. (B) FC results of MEFs, OCT4 reporter hESC line (reporter hESCs), and a nonreporter UGENT2 stem cell line (UGENT2 hESCs). Each histogram was scaled to 100% of the peak value. No autofluorescence of the UGENT2 cell line is observed. MEFs show a weak autofluorescent signal, but it is10 times lower compared with the true signal of the undifferentiated OCT4 reporter hESC line.

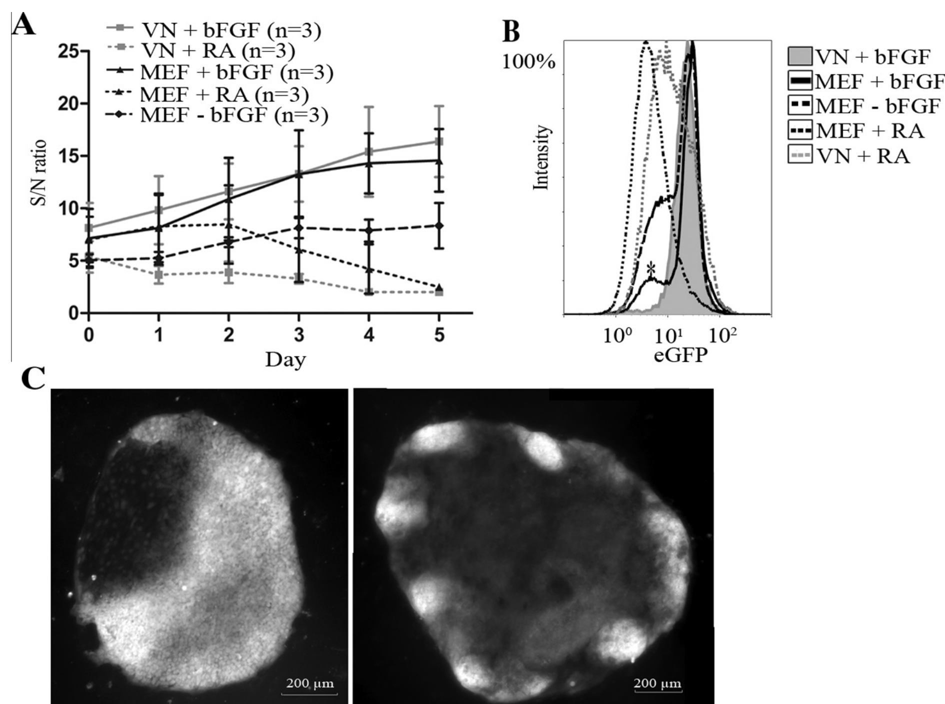

Fig.2. FM and FC results of hESCs cultured on MEFs and on VN (where n is total amount of replicates). (A) S/N ratio measured by FM of OCT4 reporter hESCs cultured on MEFs in different conditions (hESC medium with 4 ng/ml bFGF [MEF + bFGF, $n = 3$], without bFGF [MEF − bFGF, $n = 3$], and with 2 μM RA [MEF + RA, $n = 3$]) and on VN in different conditions (E8 medium [VN + bFGF, $n = 3$] and with 2 μM RA [VN + RA, $n = 5$]). In the conditions with RA, no error bars are displayed on day 5 because of both colony detachment and because the low S/N ratio makes image stitching of the colony infeasible (signal colony ≈ signal background). The experiment was carried out in triplicate ($n = 3$), and 6 colonies were monitored in each experiment. (B) FC results at day 5 of the same conditions as described in panel A. Each histogram was scaled to 100% of the peak value. Asterisk indicates MEF contamination in the plot of the OCT4 reporter hESC line on MEFs with hESC medium + 4 ng/ml bFGF. (C) Fluorescent images of an OCT4 reporter hESC colony on MEFs in hESC medium without bFGF (left) and with RA (right). A mix of high-fluorescent and low-fluorescent cells can be observed in the left image. In the right image, "islands" of high OCT4 expression can be observed.

expressed as S/N ratios (Fig. 2A). The experiment was carried out in triplicate.

As expected, the addition of 2 μM RA caused a significant decrease in S/N ratio during the time of the experiment on hESCs cultured on both MEFs and VN (Fig. 2A). This decrease was linked to a lower expression of OCT4, leading to differentiation. Of note, hESCs cultured on VN tend to detach during forced differentiation by RA, something that can be easily detected when colonies are monitored through time by FM based on their coordinates.

Because 4 ng/ml bFGF is thought to be necessary to keep hESCs on MEFs undifferentiated, culture of hESCs in the absence of bFGF would lead to differentiation, but to a slower rate than when differentiation is artificially induced as, for example, by adding RA. In Fig. 2A, the condition without bFGF has a lower S/N ratio on day 5 than the condition with bFGF on MEFs (S/N ratios = 8.34 ± 2.16 and 14.58 ± 2.98, respectively). This S/N ratio, however, is higher than when RA was added (S/N ratios = 8.34 ± 2.16 and 2.49, respectively).

Interestingly, an increase in fluorescence in the "undifferentiated" conditions with bFGF on both MEFs and VN was observed on day 5. This might be explained by (i) an increase in eGFP/cell or by (ii) the formation of multilayers (three-dimensional growth) resulting in an accumulation of fluorescent signal.

No significant differences were found in S/N ratio from hESC cultures on MEFs and those on VN for both the non-differentiating condition (with bFGF) and the differentiating condition (with RA), confirming the low impact of autofluorescence of the MEFs on FM measurements.

Flow cytometry. The above-mentioned data of FM were compared with FC measurements on the hESC population. Because of the destructive nature of this technique, analysis is performed only at the end of the experiment (day 5) (Fig. 2B). Both feeder-free and MEF-grown hESCs in the presence of bFGF retained the undifferentiated status (fluorescent signal > 10^1). Of note, a small portion of cells in the latter population had an eGFP expression between 10^0 and 10^1 (Fig. 2B, asterisk). These cells were probably MEFs, as mentioned earlier (autofluorescence between 10^0 and 10^1). The finding that the eGFP/cell remained constant in the undifferentiated conditions strongly suggests that the daily increase in fluorescence of the undifferentiated conditions as observed by FM is not due to the increase of eGFP/cell but rather is due to a multilayer effect (three-dimensional growth) resulting in accumulated fluorescent signal.

In the MEF condition without bFGF, most of the cells were still undifferentiated after a 6-day culture (fluorescent signal > 10^1), but in comparison with the MEF condition with bFGF, a significantly higher number of cells with an eGFP expression between 10^0 and 10^1 were observed. These results are in line with the FM measurements, in which it was shown morphologically that there was a mix of differentiated hESCs (low fluorescence; S/N ratio = 2.26) and undifferentiated hESCs (high fluorescence; S/N ratio = 13.75) on day 5 when bFGF was absent (Fig. 2C, left).

The conditions with RA showed a clear drop in fluorescence on day 5 that is due to differentiation (Fig. 2B). This finding is in line with the results obtained by FM (Fig. 2A). Remarkably, FM images of hESC colonies differentiated with RA revealed the existence of zones ("islands") with high accumulated fluorescence (S/N ratio = ±24) (Fig. 2C, right). This small population of high-fluorescent islands could not be discriminated using FC because these individual highly fluorescent cells were somewhat hidden in the tail of the fluorescence histogram obtained with FC (Fig. 2B).

In conclusion, when looking only at the S/N ratios measured by FM for pluripotency assessment, it is important to take into account that an increase of signal of a whole colony does not correlate with an increased eGFP signal per cell and that only a decrease in FM signal can be directly interpreted as an ongoing differentiation. A flat signal in FM can be interpreted as a hESC culture with a population of differentiating and non-differentiating cells.

Application of noninvasive monitoring of differentiation status: MEF conditioned medium

A possible application of the above-mentioned noninvasive method is a comparison of different media to test their ability to maintain hESCs in an undifferentiated state: E8 medium on VN (positive control) versus ITS-A CM versus ITS-A UCM. The difference between CM and UCM is that CM contains MEF-secreted proteins (see Materials and Methods). ITS-A was used instead of KO–SR because ITS-A contains no albumin in comparison with KO–SR, a great advantage when subsequent MS analysis of the media is envisioned. To our knowledge, this is the first time that ITS-A-containing MEF CM without KO–SR was evaluated for its ability to keep hESCs pluripotent.

On top of validating the impact of MEF-secreted proteins, the influence of the addition of bFGF (CM+ and UCM+ contain bFGF, whereas it is absent in CM− and UCM−) was also verified. Besides OCT4 analyses, cell number could be determined as well with FC.

eGFP expression assessed by fluorescence microscopy

After a 6-day culture, surprisingly no significant difference in eGFP expression could be observed between the positive control (E8) and the other conditions (CM+, CM−, UCM+, and UCM−) (Fig. 3A), suggesting that none of the tested supplements significantly downregulated OCT4 expression during short-term culture. Morphological assessment of the colonies showed a more or less uniform distribution of the fluorescence in the colony in all conditions, indicating a comparable multilayer formation if all cells have the same OCT4 expression (confirmed with FC).

eGFP expression assessed by flow cytometry

FC was used to validate the results of FM at the end of the experiment (Fig. 3B and C). In addition to OCT4 expression at day 5 (by means of x-mean of the green channel; Fig. 3B), the amount of cells was also counted by using flow count beads as a reference (Fig. 3C). Both values are expressed as the logarithmically normalized ratio with respect to E8 (positive control). As seen in Fig. 3B, a slight decreasing trend (not significant) in OCT4 expression at the single-cell level can be observed over the different conditions at day 5, confirming FM results. In contrast, the total cell number at the end of the experiments was on average higher in CM compared with UCM, with statistical significance found only between CM− and UCM− (P value <0.05).

Confluence

This difference in cell number between CM and UCM was verified by assessing the confluence of colonies by use of a Celigo imaging cell cytometer (Fig. 3D). Less confluence could be observed in the conditions with UCM, confirming the results obtained with FC.

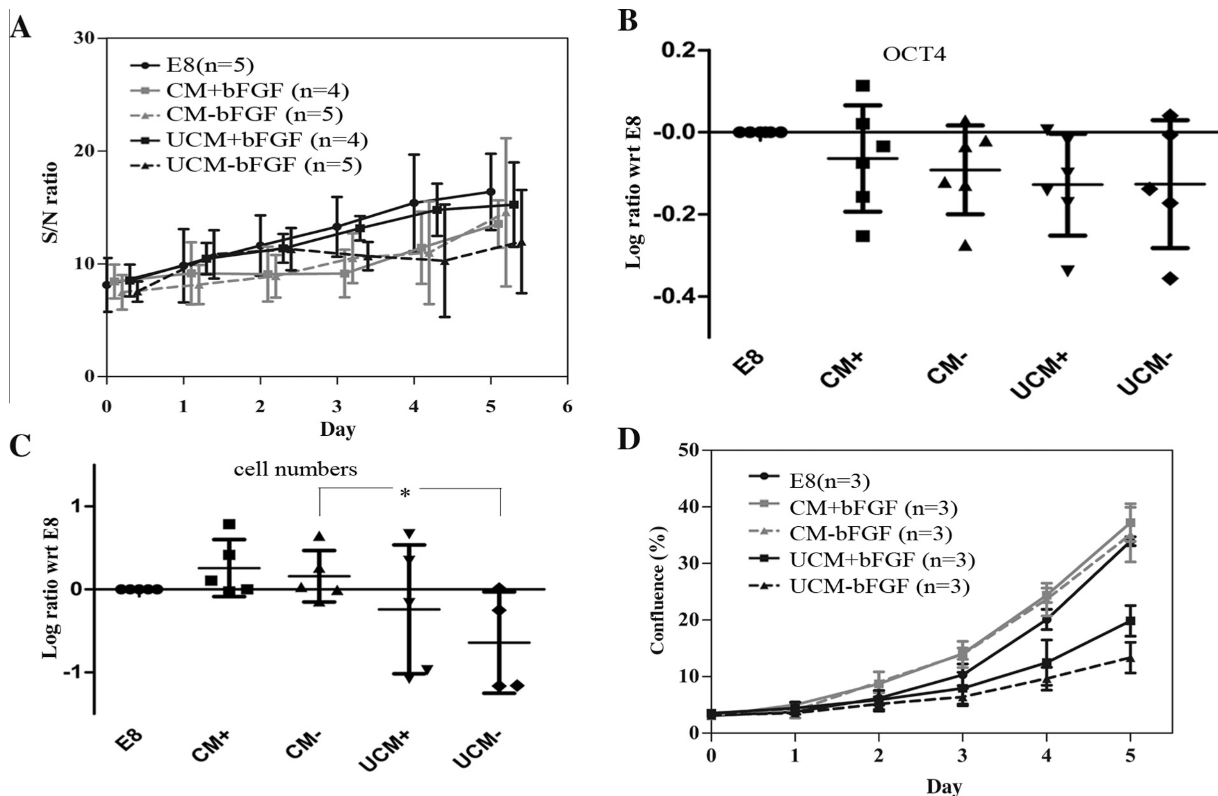

Fig.3. Influence of different media on hESC pluripotency and cell growth measured by means of FM and FC. The experiment was carried out in triplicate or more (n = 3, 4, or 5), and 6 colonies were monitored in each experiment. (A) S/N ratio after FM of OCT4–eGFP knock-in hESCs on VN in combination with different media (E8, CM [with and without bFGF], and UCM [with and without bFGF]) for 6 days. (B) OCT4 expression by means of x-mean of the above-mentioned conditions determined with FC on day 6. Results are expressed as the log ratio with respect to (wrt) E8 (positive control) for normalized representation. (C) Cell number of the above-mentioned conditions determined with FC after 6 days. Flow count beads were used to assess absolute count concentration. Results are expressed as the log ratio with respect to E8 (positive control) for normalized representation. *P value <0.05. (D) Confluence (%) determined by means of the Celigo cytometer of the same above-mentioned conditions for 6 days.

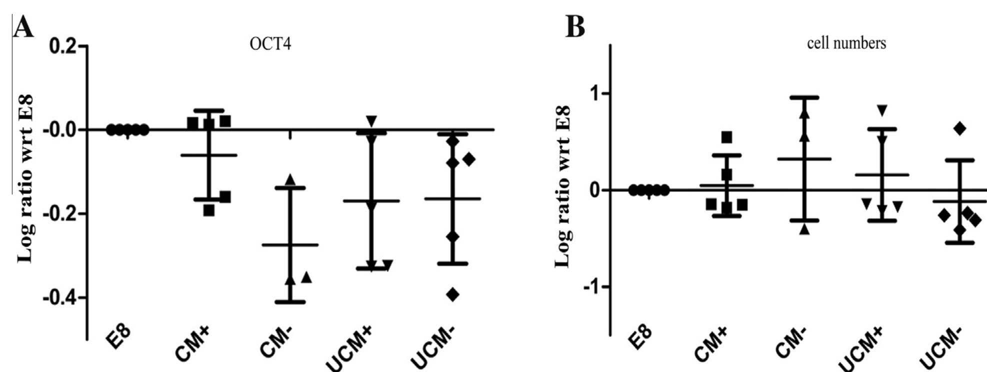

Fig.4. Influence of stress induced by measurement on FM. FC results on day 6 of the different media as mentioned in Fig. 3 are shown. No FM measurement was carried out. Results are expressed as the log ratio with respect to (wrt) E8 (positive control) for normalized representation. (A) OCT4 expression by means of *x*-mean. (B) Cell numbers.

Stress

We noticed that, especially in UCM media, reproducibility of the experiments was very low. In both FM and Celigo, however, some stress is induced by removal of the medium before measurement and by the lack of a controlled environment in the apparatus used during these experiments (temperature, CO_2, and O_2). We hypothesize that this stress caused the lack of reproducibility in UCM cultures. No stress was induced by analyzing hESCs only at the end of the experiment (on day 5) with FC without intermediate FM or Celigo measurements (no stress during time of the experiment), and these results are displayed in Fig. 4A (OCT4 expression) and B (cell numbers). Note that media were replaced throughout the time course of the experiment and that detached cells were removed each day, as was the case in the earlier described experiments.

Importantly, no significant influence of stress on OCT4 expression could be observed. When comparing the cell number, however, it is clear that the differences in cell number in the different conditions (Fig. 3C) is strongly reduced when hESCs are grown without stress. This stress-caused effect can probably be avoided by using autofluorescence-free medium in combination with a controlled environment.

Discussion

The goal of this study was to validate an easy and nondestructive method to follow up pluripotency (by expression of OCT4) and morphology of hESCs. To our knowledge, this is the first detailed description of such a method for evaluating the (non)differentiation status of hESC. It is based on the use of a commercially available OCT4–eGFP knock-in hESC line (WiCell Research Institute) in combination with FM. Method validation and cell number assessment were accomplished by means of FC as the gold standard. Therefore, no special live cell imaging instruments are required when implementing this method.

As a measure of OCT4 expression, colony S/N ratio (=densitometric $mean_{colony}$/densitometric $mean_{background}$) and single-cell *x*-mean of the fluorescence were measured by FM and FC, respectively. Using FM, we found a surprising daily increase in S/N ratio of hESC colonies in the undifferentiated conditions (+bFGF). When measuring OCT4 expression at the single-cell level with FC, this increase in fluorescence was not observed (same eGFP/cell during the experiment). Therefore, increased S/N ratio of whole colonies seen by FM is likely due to three-dimensional growth (=multilayer effect). Although FM can be used for following up the OCT4 expression of hESC colonies, one should keep in mind that converting these images into a single number (S/N ratio) will, by definition, result in a loss of information content. Before implementing FM as a nondestructive screening tool, S/N ratio values first need to be compared with additional FC measurements to examine the OCT4 expression at the single-cell level.

However, FM, and not FC, can assess changes in colony homogeneity and morphology. This was clearly illustrated by islands of high fluorescence in RA-differentiated hESC colonies. These islands expressed high amounts of eGFP and, thus, high amounts of OCT4, suggesting the existence of small remaining populations of non-differentiated cells after 6 days of RA-induced differentiation. These cells were somewhat "buried" in the tail of the FC histogram and, thus, would never be detected by this technique. Costaining with other germ line markers is needed to elucidate the origin of these islands, but this lies outside the scope of this study.

Of note, we tried to define colony homogeneity by following up the standard deviation (SD) of the colony during the time of the experiment with FM (colony homogeneity cannot be assessed by FC (only single-cell analysis)). In theory, a polymorph colony (regions of high- and low-fluorescent areas) will have a higher SD than a uniform colony and the formation of a polymorph colony will lead to an increase in SD during the time of the experiment. Different SD calculations were tested, but none showed in full the polymorphism of the colony in comparison with the morphological images taken by FM (data not shown).

Although FC will still be needed to assess information at the single-cell level, FM is unique in providing daily information about the distribution of OCT4 expression in different colonies in a nondestructive way that no other technique could accomplish up to now, thereby giving new insights into how cells will respond to different stimuli in terms of hESC differentiation. For defining lineage commitment, however, one must still stain with other markers such as SSEA-1 and GATA4.

Once FM measurements have been essayed against FC, our method can be used to, for example, analyze the effect of different compounds (small molecules and proteins) on hESC pluripotency, morphology, and cell growth. This application was tested by observing differences in colony growth in media that were conditioned by MEFs prior to culture of the hESCs. Here, FM was used to monitor the OCT4–eGFP knock-in hESC line in both CM and UCM with and without the addition of bFGF. The commercially available Essential 8 medium was used as a positive control [12]. Our results indicated that all media can retain the hESCs in their undifferentiated status for at least 6 days. These findings were corroborated by FC measurements at day 6. FC (after FM analysis) and Celigo, however, did show a higher cell number at day 6 with hESCs grown in CM compared with UCM. This observation was not seen by analyzing hESCs with FC alone (no FM analysis). FM analysis and Celigo will induce some stress that can probably be avoided by using an incubator system with autofluorescent-free

medium (e.g., riboflavin is a well-known autofluorescent substance) [13]. More colonies can then also be monitored. Indeed, software for automatic measurement of colony fluorescence is already under construction by companies such as Brooks (Celigo system). However, because of the difference in half-life between eGFP (half-life $\geqslant$ 24 h) and OCT4 (half-life in mice = 6–8 h), the fluorescent signal follows the OCT4 expression and only the absence of a signal can directly be interpreted as a lack of OCT4 in the cell [14–16].

Of note, a disadvantage of the FM method described here is that for larger colonies, several images need to be taken of each colony, which subsequently need to be stitched to visualize the whole colony in a single image. In our hands, a lower magnification objective (2.5$\times$ instead of 10$\times$) could not be used because this led to lower fluorescent signals of the colonies, resulting in unusable S/N ratios. Stitching results in increased handling time, thereby increasing the stress that these cells need to undergo. This is because media needed to be removed to avoid autofluorescence, and the microscope used during these experiments was not equipped with an incubation chamber.

Conclusion

We have described in detail a method for the use of FM to monitor the (non)differentiation status of hESC colonies by using a commercially available OCT4–eGFP knock-in hESC line. We focused on the pitfalls and benefits of this noninvasive screening method by testing it against FC as the gold standard. FM is capable of following the (non)differentiation status of different colonies for several days but has the added value of observing morphological changes indiscernible by FC. Together with complementary FC data, such as cell number and eGFP/cell, this provides an additional dimension in defining the (non)differentiation status of a culture.

This optimized FM setup can be used to analyze the impact of different media on the (non)differentiation status of the hESC line growing on VN.

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