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Live Fluorescent RNA-Based Detection of Pluripotency Gene Expression in Embryonic and Induced Pluripotent Stem Cells of Different Species

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ABSTRACT

The generation of induced pluripotent stem (iPS) cells has successfully been achieved in many species. However, the identification of truly reprogrammed iPS cells still remains laborious and the detection of pluripotency markers requires fixation of cells in most cases. Here, we report an approach with nanoparticles carrying Cy3-labeled sense oligonucleotide reporter strands coupled to gold-particles. These molecules are directly added to cultured cells without any manipulation and gene expression is evaluated microscopically after overnight incubation. To simultaneously detect gene expression in different species, probe sequences were chosen according to interspecies homology. With a common target-specific probe we could successfully demonstrate expression of the *GAPDH* house-keeping gene in somatic cells and expression of the pluripotency markers *NANOG* and *GDF3* in embryonic stem cells and iPS cells of murine, human, and porcine origin. The population of target gene positive cells could be purified by fluorescence-activated cell sorting. After lentiviral transduction of murine tail-tip fibroblasts *Nanog*-specific probes identified truly reprogrammed murine iPS cells *in situ* during development based on their Cy3-fluorescence. The intensity of *Nanog*-specific fluorescence correlated positively with an increased capacity of individual clones to differentiate into cells of all three germ layers. Our approach offers a universal tool to detect intracellular gene expression directly in live cells of any desired origin without the need for manipulation, thus allowing conservation of the genetic background of the target cell. Furthermore, it represents an easy, scalable method for efficient screening of pluripotency which is highly desirable during high-throughput cell reprogramming and after genomic editing of pluripotent stem cells. STEM CELLS *2015;33:392–402*

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INTRODUCTION

A major progress toward a potential regenerative therapy was accomplished with the discovery that somatic cells can be reprogrammed to a pluripotent stage by the overexpression of a cocktail of specific transcription factors. Murine fibroblasts were reprogrammed into so-called induced pluripotent stem (iPS) cells a couple of years ago [1]. Meanwhile iPS cells have successfully been established from other species including humans [2], rats [3], monkeys [4], dogs [5], sheep [6], and pigs [7].

iPS cells serve as a powerful cellular tool in different research areas. They have been used to unravel disease-related molecular mechanisms (disease modeling) in particular for genetically inherited diseases [8–10]. Since they harbor the patient-specific genetic background they allow a customized screening of the efficiency of drugs that might be used as therapeutic agents [11–13]. Another promising research field is regenerative therapy, as iPS cells can be produced in almost unlimited cell numbers in automated bioreactors [14]. Transplanted cardiac tissue derived from human iPS cells has been shown to improve the cardiac function in a porcine model of ischemic cardiomyopathy [15]. For such an application it is essential to provide safe transplantable material which is depleted of pluripotent cells in sufficient amounts as quickly as possible.

After the establishment of potential iPS clones a major challenge remains the laborious and time-consuming characterization of these lines and the proof that they are indeed fully reprogrammed to a pluripotent stage. However, in most cases, a definite verification of gene expression is impossible on living cells. The detection of endogenous pluripotency markers such as *OCT4*, *SOX2*, *NANOG*, *GDF3*, and *REX1* requires fixation of the cells prior to staining [16–18] or is accomplished only

after lysis by an RT-PCR analysis [17, 18]. With such a manipulation, the cells are lost for further culture. Therefore, detection of pluripotency markers on live cells is highly desirable. This is feasible and routinely performed for cell membrane-based antigens such as TRA-1–60 or SSEA4 [18] although the antibodies might nevertheless perturb the cells [19] and these "early pluripotency markers" do not allow discrimination of terminally stable reprogrammed iPS cells. Also for alkaline phosphatase (AP), which is another early pluripotency marker mostly assessed on fixed cells [16], a fluorescent reporter dye ("AP Live Stain") has been lately developed for live screening [20]. In addition, a fluorescent small molecule named CDy1 has been reported to specifically stain living embryonic stem cells (ESCs) and iPS cells [21]. However, even with these approaches the screening is based on and restricted to a single factor.

The great potential of iPS cells in many research areas has raised the demand to generate, characterize, and expand these cells by high-throughput technologies. Major efforts have been undertaken to define appropriate culture conditions [22] and to establish procedures which allow the identification of properly reprogrammed iPS cells by specific markers or genetic signatures [23–25]. Our approach provides a novel component which should be helpful in the identification of truly reprogrammed iPS cells during high-throughput screening. We describe the use of a novel RNA-based technique (SmartFlare™ Probe) that allows an easy and rapid detection of gene expression of any desired pluripotency marker in live cells regardless of its subcellular location. Using fluorescent SmartFlare™ reporter probes with sequences homologous to different species we could reliably detect expression of *NANOG* and *GDF3* in live murine ESCs as well as iPS cell colonies *in situ* of human, murine, and porcine origin. Finally, we provide evidence that our approach is suitable to screen and visually identify successfully reprogrammed iPS colonies.

Materials and Methods

Culture of Established Cell Lines and Primary Fibroblasts

Human 293 embryonic kidney cells (CRL-1573, ATCC, Manassas, VA, www.atcc.org) and murine tail tip fibroblasts (TTFs) from collagenase2/DNaseI-digested tail tips (37°C, 45 minutes) from C57Bl/66 and Nkx2.5 CE eGFP mice [26] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (293 cells) or 10% fetal calf serum (TTFs), 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin/100 μg/ml streptomycin. Porcine and ovine primary fibroblasts were isolated from adipose tissue according to the protocol recently described by Gu et al. [27]. Briefly, adipose tissue was cut into small pieces and incubated for 2.5 hours at 37°C with collagenase 2 (2 mg/ml, Worthington, Lakewood, NJ, www.worthington-biochem.com). Cells were centrifuged, washed with phosphate buffered saline, filtered through a 70 μm filter, and cultured in DMEM supplemented with 10% fetal calf serum, 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin/100 μg/ml streptomycin as monolayers.

Culture of Murine ESCs

Murine V6.5 ESCs and the recently established Nkx2.5 cardiac enhancer eGFP ESC line (Nkx2.5 CE eGFP ES) [26] were grown on a monolayer of mitomycin-arrested murine embryonic fibroblasts (MEFs) with murine ESC medium consisting of DMEM supplemented with 4.5 g/l glucose, 15% fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml leukemia inhibitory factor (Millipore, Billerica, MA), and 100 U/ml penicillin/100 μg/ml streptomycin. Cells were supplemented daily with new medium. Subpassaging of cells was done by trypsinization.

Generation and Culture of Murine, Human, and Porcine iPS Cells

Primary murine TTFs from Nkx2.5 CE eGFP transgenic mice [26] were cultured until passage 3 and then transduced with lentiviruses containing the doxycycline-inducible pHAGE2-Tet-STEMCCA, a polycistronic vector with *Oct3/4*, *Sox2*, c-*Myc*, and *Klf4* sequences [28] and pFudeltaGW-rtTA with a reverse tetracyclin transactivator driven by a ubiquitous CMV promotor (rtTA). Doxycycline (1 μg/ml) was added starting at day 2 after transduction. On day 3, transduced TTFs were replated on a MEF feeder layer. SmartFlare™ Probes specific for *Nanog* or *Gdf3* were added on day 9 when visible iPS colonies had emerged. Two days later fluorescent colonies were picked and expanded in the presence of gradually reduced amounts of doxycycline. Murine iPS cells were supplemented daily with fresh murine ESC medium and subpassaged by trypsinization.

The human iPS line C6 was generated by reprogramming skin fibroblasts from a healthy donor with the CytoTune® iPS-reprogramming kit (Invitrogen, Darmstadt, Germany, www.lifetechnologies.com) according to the manufacturer's recommendation. Primary porcine adipose-derived fibroblasts were reprogrammed into iPS cells with a lentivirus containing the polycistronic phu STEMCCA vector (Millipore, Darmstadt, Germany, www.merckmillipore.de). Human and porcine iPS cells were cultured on mitomycin-arrested MEFs in human iPS medium consisting of DMEM/Ham's F-12 supplemented with 20% knockout serum (Invitrogen), 2 mM L-glutamine, 0.1 mM nonessential amino acids (GIBCO, Darmstadt, Germany, www.lifetechnologies.com), 10 ng/ml basic fibroblast growth factor (Peprotech, Hamburg, Germany, www.peprotech.com), and 100 U/ml penicillin/100 μg/ml streptomycin. Fresh human iPS medium was added daily. Cells were subpassaged by mechanical dissection.

Selection of SmartFlare™ Probes Capture Sequences and Reconstitution of Probes

For the design of appropriate reporter strands sequences, we first performed a CLUSTAL alignment of human, murine, and porcine *GAPDH*, *NANOG*, and *GDF3* sequences. For *GAPDH*, the ovine sequence was included in addition. The desired capture strand sequences of 26 or 27 bp length were chosen according to interspecies homology. Two different probes were designed for *GAPDH* (GAPDH-SF2 and SF3) and *GDF3* (GDF3-SF1 and SF-2) while three sequences were selected for *NANOG* (NANOG-SF1, SF2, and SF3). The alignment and the location of the reporter sequences are depicted in Supporting Information Figure S1. Lyophilized SmartFlare™ Probes were reconstituted with sterile aq.bidest. to a final concentration of 100 nM and stored at room temperature in the dark until further use.

To identify adequate reporter sequences, the complete sequence of a given target gene (coding and noncoding regions) was scanned by a software tool. The software considers parameters such as the melting temperature (Tm), the GC

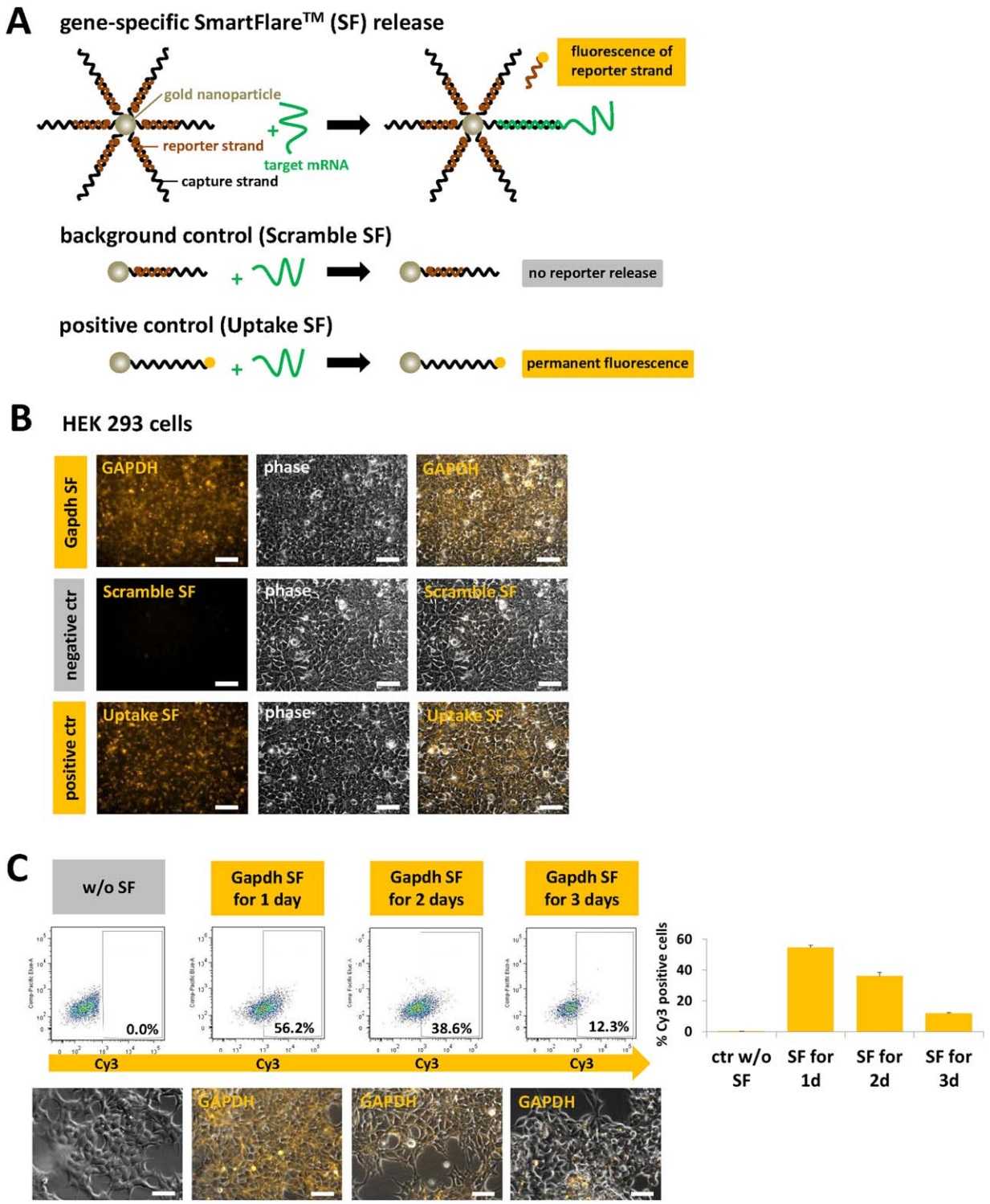

Figure 1. Detection of *GAPDH* expression in human 293 cells. **(A):** Principle of detection of gene expression by SmartFlare™ Probes. **(B):** Human 293 embryonic kidney cells. Fluorescence was recorded 24 hours after the addition of SmartFlare™ Probes. Scale bars represent 50 μm. **(C):** Kinetics of SmartFlare™ fluorescence in human 293 cells. For each time point representative fluorescence-activated cell sorting plots and pictures are shown. The values in the bar chart represent the mean of two independent cultures ± SE.

content, or potential self-complementarity. Based on these results, it predicts whether or not a certain sequence will be functional as a SmartFlare™ Probe in live cells. All information of previous designs is fed into the program to improve its accuracy when searching for novel reporter sequences.

Live Staining of Cells

SmartFlare™ Probes were diluted with phosphate buffered saline to the desired concentration and were then directly added to the culture medium at 5% (vol/vol) yielding a final concentration of 100 pM (fibroblasts) or 400 pM (ESCs and

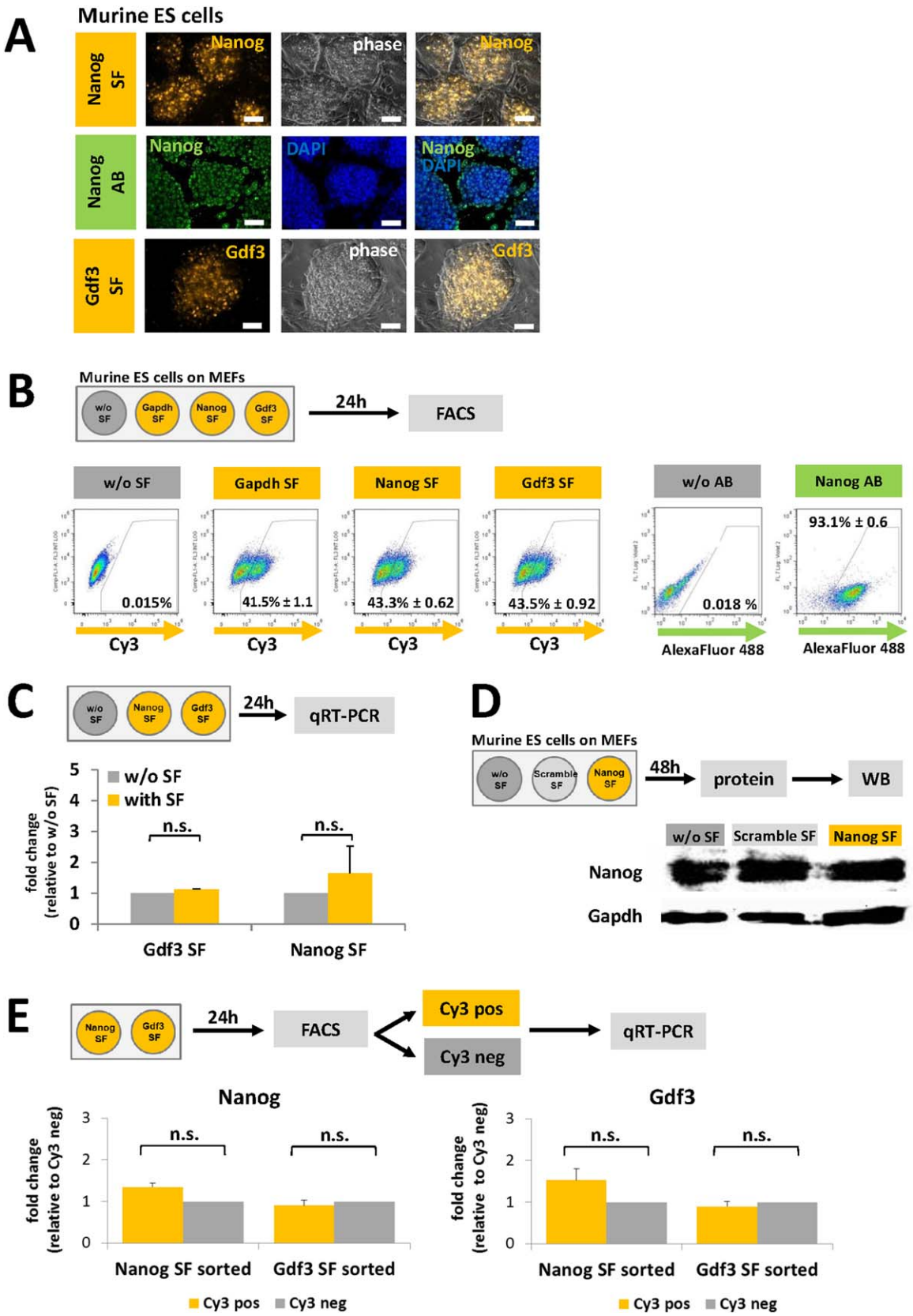

Figure 2.

iPS cells), respectively. In each experiment, two controls were included: a construct which does not recognize any cellular sequence served as a control to determine the background (scramble SmartFlare™ Probe). A positive control (uptake SmartFlare™ Probe) which permanently fluoresces provided the information that the SmartFlare™ particles were incorporated by the target cell type. If not stated otherwise the fluorescence was evaluated on the next day with an Axiovert 200M fluorescence microscope (Carl Zeiss, Oberkochen, Germany, www.zeiss.de). Pictures of fluorescent cells were taken with the same shutter-speed for each cell type.

Immunocytochemistry

Cells were grown on cover slides and fixed with acetone for 10 minutes at −20°C and permeabilized with 0.1% Triton X-100 in phosphate buffered saline for 10 minutes. Unspecific binding was blocked by incubation with 5% goat serum for 30 minutes, followed by a 1 hour incubation with rabbit IgG-anti-Nanog (1:250, ab80892, Abcam, Cambridge, U.K., www.abcam.com). Thereafter, AlexaFluor488 labeled secondary goat anti-rabbit IgG antibody (1:500, ab150077, Abcam) was added for 1 hour. All incubations were performed at room temperature. UltraCruz™ Mounting Medium with DAPI (sc-24941, Santa Cruz Biotechnology, Heidelberg, Germany, www.scbio.de) was added and slides were sealed with coverslips.

Fluorescence-Activated Cell Sorting Analysis of SmartFlare™ Labeled Cells

After addition of SmartFlare™ Probes, single-cell suspensions were prepared at the indicated time. Cells were trypsinized, washed in phosphate buffered saline/0.5% bovine serum albumin, and the cell pellet was resuspended in ice-cold phosphate buffered saline/0.5% bovine serum albumin/2 mM EDTA. To exclude dead cells propidium iodide was added at 2 μg/ml.

Detection of Green Fluorescent Protein-Positive Cardiac Precursor Cells

Murine Nkx2.5 CE eGFP iPS cells were grown for 2 days on gelatin-coated dishes in Iscove's modified Dulbecco's medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 0.1 mM monothioglycerol, leukemia inhibitory factor (10^3 U/ml), and 100 U/ml penicillin/100 μg/ml streptomycin to deplete MEFs. Cells were trypsinized and cultured upside down as hanging drops (2,000 cells/11 μl drop) in differentiation medium (Iscove's modified Dulbecco's medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 50 μg/ml ascorbic acid, 0.1 mM monothioglycerol, and 100 U/ml penicillin/100 μg/ml streptomycin). After 2 days, the dishes were flooded with differentiation medium. On day 7, single-cell suspensions were prepared by incubation with collagenase type 2/DNase I for 45 minutes at 37°C. After resuspension in ice-cold phosphate buffered saline/0.5% bovine serum albumin/2 mM EDTA, the frequency of green fluorescent protein (GFP)-positive cardiac precursor cells was determined by fluorescence-activated cell sorting (FACS) analysis.

Assessment of Gene Expression by qRT-PCR

Total RNA was isolated using the peqGold total RNA Kit according to manufacturer's instructions (Peqlab, Erlangen, Germany, www.peqlab.de) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Amplification was performed on a LightCycler 1.5 (Roche Diagnostics, Mannheim www.roche.de) using the following conditions: 95°C for 15 minutes to activate *Taq* polymerase, followed by 40 cycles of 95°C for 10 seconds, annealing at 60°C for 20 seconds, and synthesis at 72°C for 20 seconds. The sequences of the used primers are indicated in Supporting Information Table S1.

Statistics

The significance of differences of gene expression was evaluated using the unpaired Student's *t* test. A value of $p < .05$ was considered to be statistically significant.

Results

Detection of Intracellular GAPDH Gene Expression in Live Cells of Different Species Using a Common SmartFlare Reporter Probe

The SmartFlare™ system comprises a central gold nanoparticle to which gene-specific double-stranded DNA oligonucleotides are attached (Fig. 1A). They consist of a longer RNA capture strand and a shorter Cy3-labeled complementary reporter strand. In the original state, the fluorescence is quenched by the gold particle. Upon binding of a specific target RNA at the capture strand the reporter strand is displaced and emits the fluorescence. The unspecific background fluorescence is determined by a background control (Scramble SmartFlare™ Probe) which does not recognize any sequence in a eukaryotic cell. The positive control (Uptake SmartFlare™ Probe) is always "on" within a living cell (Fig. 1A). To establish and validate the SmarteFlare™ method for detection of intracellular gene expression, we first designed a SmartFlare™

Figure 2. Analysis of *Nanog* and *Gdf3* expression in murine V6.5 ESCs. **(A):** Detection of *Nanog* and *Gdf3* expression by SmartFlare™ Probes and immunocytochemical staining of Nanog protein. Fluorescence was recorded 24 hours after the addition of SmartFlare™ Probe. Scale bars represent 50 μM. **(B):** FACS analysis to detect gene expression and Nanog protein. Analyses were performed 24 hours after the addition of SmartFlare™ Probes. Representative FACS plots are shown. Values represent the mean of three independent experiments ± SE. **(C):** The addition of SmartFlare™ Probes does not significantly change the expression of *Nanog* and *Gdf3*. Total RNA was extracted from untreated cells or cultures to which *Nanog*- or *Gdf3*-specific SmartFlare™ Probes were added. Twenty-four hours after application of SmartFlare™ Probes, total RNA was extracted, reverse transcribed into cDNA, and gene expression was determined by qRT-PCR analysis. Values represent the mean of three independent experiments ± SE. Samples were normalized against an internal control (β-actin). **(D):** Addition of *Nanog*-SmartFlare™ Probes does not affect the Nanog protein level. Cells were lysed 48 hours after the addition of SmartFlare™ Probes and Nanog and Gapdh protein levels were detected by Western blot. **(E):** Expression of *Nanog* and *Gdf3* in Cy3-positive and -negative cells. SmartFlare™ Probes were added and cells were sorted by FACS into Cy3 positive and negative fractions after 24 hours. Total RNA was extracted, reverse transcribed into cDNA, and expression of *Nanog* and *Gdf3* was determined by qRT-PCR. Values represent the mean of three independent experiments ± SE. Samples were normalized against an internal control (β-actin). Abbreviations: ESCs, embryonic stem cells; FACS, fluorescence-activated cell sorting; AB, antibody; w/o, without, WB, Western blot; SF, SmartFlare™ Probe.

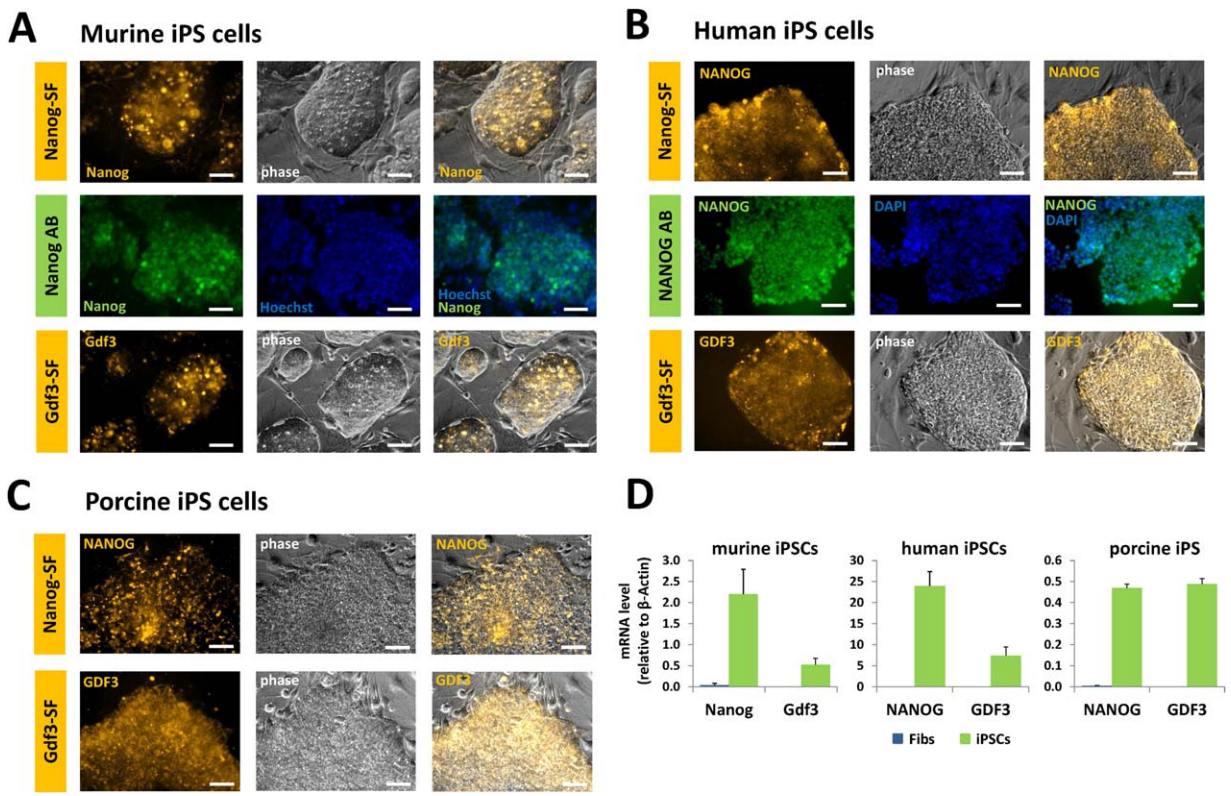

Figure 3. SmartFlare™ Probes reliably detect the expression of pluripotency markers *NANOG* and *GDF3* in iPS cells of different species. **(A–C):** Detection of *NANOG* and *GDF3* expression by SmartFlare™ Probes and immunocytochemical staining of Nanog protein in murine (A), human (B), and porcine (C) iPS cells. Fluorescence was recorded 24 hours after the addition of SmartFlare™ Probes. Immunocytochemical staining for NANOG was performed in parallel. Scale bars represent 50 μM. **(D):** qRT-PCR analysis of expression of *NANOG* and *GDF3* in untransfected fibroblasts (fibs, dark blue bars) and iPS cells (iPSCs, green bars). Values represent the mean of three independent experiments ± SE. Samples were normalized against an internal control (β-actin). Abbreviation: iPS, induced pluripotent stem; AB, antibody; SF, SmartFlare™ Probe.

Probe specific for the housekeeping gene *GAPDH* choosing a sequence of the *GAPDH* mRNA that is homologous between different species (Supporting Information Fig. S1). Twenty-four hours after the application of the *GAPDH* probe to human 293 embryonic kidney fibroblasts Cy3-fluorescence was readily detectable. Its intensity was similar to the one of the uptake control (Fig. 1B). In contrast, hardly any fluorescence was detectable in the background control (Fig. 1B). Most importantly, the addition of SmartFlare™ Probes did not affect the proliferation of 293 and Nkx2.5 CE eGFP ESCs even when applied at very high concentrations (Supporting Information Fig. S2). Analogous results were SmartFlare™ based fluorescence was obtained in primary cultures of murine, porcine, and ovine origin (Supporting Information Fig. S3), suggesting the feasibility of the SmartFlare™ approach for detection of intracellular gene expression across species with a common specific probe also in primary cell cultures.

In order to determine the intrinsic labeling kinetics of SmartFlare™ Probes, we tracked the fluorescence of 293 cells after addition of the *GAPDH* probe over a longer time period (Fig. 1C). More than 50% of 293 cells showed a clear fluorescent signal in a FACS analysis 1 day after the addition of the probe. The proportion of Cy3-positive cells dropped to approximately 40% after 48 hours but was still evident after 3 days (Fig. 1C). Therefore, the ideal time to microscopically evaluate the Cy3-fluorescence appears to be 24 hours after the addition of the SmartFlare™ Probe.

Detection of Pluripotency Gene Expression in Murine ESCs by SmartFlare™ Probes

The expression of *NANOG* and *GDF3* in pluripotent cells is known for almost a decade [29]. Therefore, we used the well-established murine V6.5 ESC line to investigate the functionality of the SmartFlare™ Probes specific for these genes. We first analyzed the expression of *Nanog*. A bright fluorescent signal was detected with a Nanog-specific SmartFlare™ Probe (Fig. 2A). Noteworthy, the fluorescence was restricted to the ESCs without any cross-signal in the embryonic fibroblast feeder layer. Immunocytochemical staining confirmed the expression of Nanog protein in these cells (Fig. 2A). Application of the *Gdf3*-specific probe to the same cells resulted in a specific fluorescent signal restricted to the murine ESC colonies (Fig. 2A). We then used the same SmartFlare™ Probes on the murine ESC line Nkx2.5 cardiac enhancer (CE) eGFP, which has a proven status of pluripotency [26] and a similar specific fluorescence could be detected on these cells for *Nanog* and *Gdf3* (Supporting Information Fig. S4). These experiments clearly show the functionality of the SmartFlare™ Probes for the specific detection of *Nanog* and *Gdf3* in two independent murine ESC lines.

Subsequently, we analyzed the frequency of Cy3 positive V6.5 ESCs 24 hours after one single application of different SmartFlare™ Probes by flow cytometry. More than 40% of the ESCs were stained by the *Gapdh*-specific SmartFlare™ Probe (Fig. 2B). The application of an uptake control in

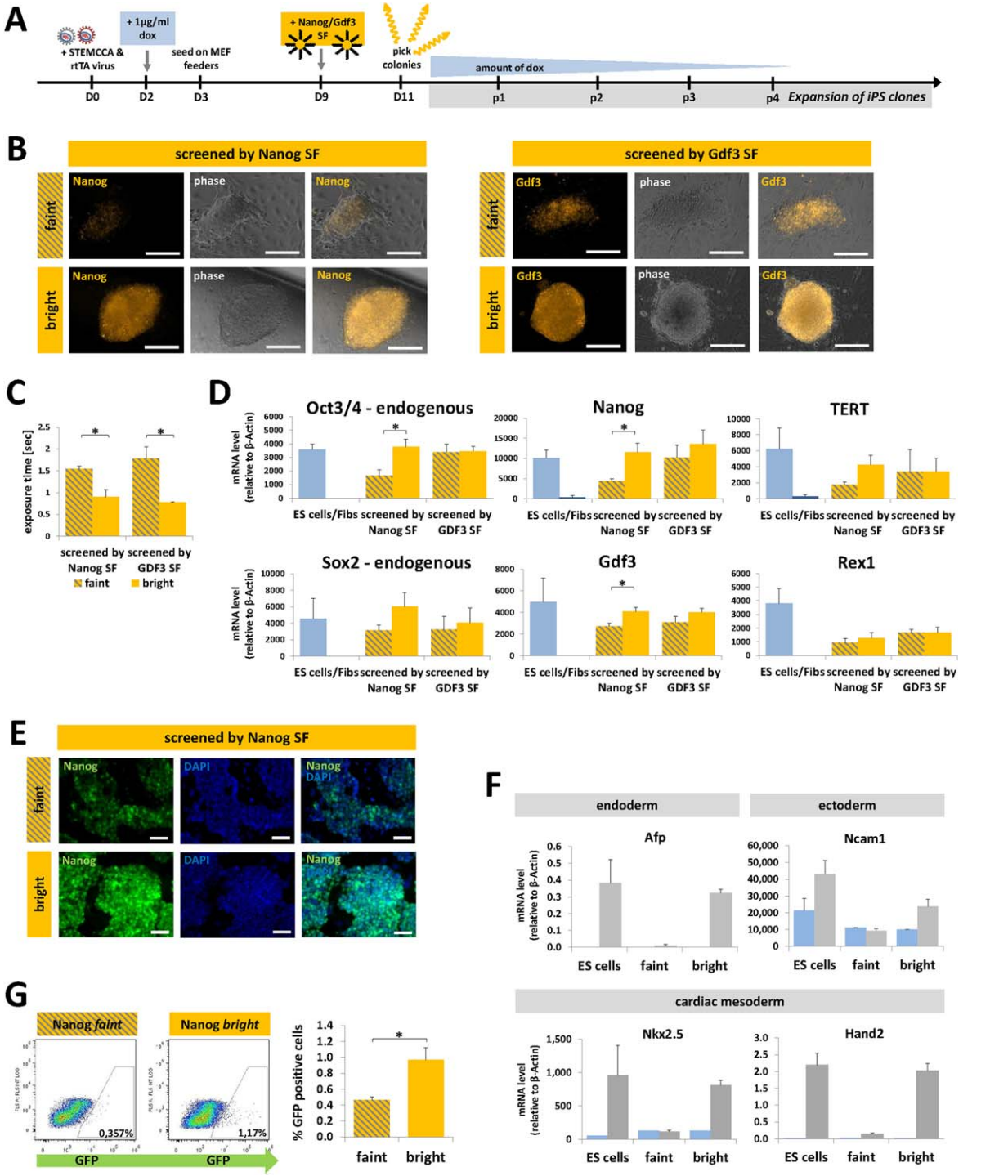

Figure 4. SmartFlare™ Probes allow live screening for successfully reprogrammed developing murine induced pluripotent stem (iPS) colonies. **(A):** Schematic of murine iPS generation. **(B):** Representative examples of "faint" and "bright" expression of *Nanog* and *Gdf3* in developing murine iPS colonies. Pictures were taken on day 10, 1 day after the addition of SmartFlare™ Probes. Scale bars represent 50 µM. **(C):** Exposure time to record fluorescence in faint (hatched bars) and bright (yellow bars) fluorescing iPS colonies. *, $p < .05$. **(D):** qRT-PCR analysis of expression of pluripotency markers at passage 2 after picking in expanded faint and bright fluorescing iPS colonies. ESCs: light blue bars, fibroblasts (fibs): dark blue bars, faint fluorescing colonies: hatched bars, bright fluorescing colonies: yellow bars. Samples were normalized against an internal control (β-actin). *, $p < .05$. **(E):** Immunocytochemical analysis of Nanog protein in faint (top panel) and bright fluorescing (bottom panel) iPS clones. **(F):** Undirected differentiation of faint and bright fluorescing iPS clones to obtain cells of all three germ layers. Undifferentiated cells: light blue bars, differentiated cells: gray bars. Values represent the mean of three independent experiments ± SE. Samples were normalized against an internal control (β-actin). **(G):** Frequency of cardiac precursor cells (CPCs) upon differentiation of faint and bright fluorescing colonies. CPCs were identified by the appearance of GFP fluorescence. Representative fluorescence-activated cell sorting plots are shown. *, $p < .05$. The CPC frequency was determined in three independent experiments. Abbreviations: ESCs, embryonic stem cells; GFP, green fluorescent protein; SF, SmartFlare™ Probe.

parallel cultures resulted in a similar frequency of Cy3-positive cells (data not shown). ESC samples treated with *Nanog*- or *Gdf3*-specific probes gave comparable amounts of Cy3-positive cells (Fig. 2B), expectable since pluripotency genes are likewise ubiquitously expressed in ESCs. While SmartFlare™ Probes were added to three dimensionally growing ES colonies in the culture dishes, a suspension of single permeabilized cells had to be prepared for the concomitant detection of Nanog protein in a FACS analysis. Most probably due to this manipulation Nanog protein was detectable in almost every cell (Fig. 2B).

Next we tested whether the addition of a SmartFlare™ Probe would influence the mRNA or protein level of the targeted gene. Compared to untreated control cells no significant differences of *Gdf3* ($p = .8$) or *Nanog* mRNA ($p = .515$) levels were seen in V6.5 ESC samples 24 hours after the addition of SmartFlare™ Probes (Fig. 2C). Furthermore, the level of Nanog protein in V6.5 cells remained virtually unchanged after the addition of a Nanog-specific SmartFlare™ Probe compared to cells that received the scramble SmartFlare™ (Fig. 1A) or were left untreated (Fig. 2D). Finally, we compared the expression of both pluripotency genes in Cy3-positive and -negative cell fractions after cell sorting. In the case of *Nanog*, slightly higher amounts of *Nanog* mRNA were detected in Cy3-positive cells while the expression level of *Gdf3* was pretty similar in both fractions (Fig. 2E).

Detection of Pluripotency Gene Expression in iPS Cells of Murine, Human, and Porcine Origin

Having established the interspecies cross-reactivity and the successful detection of *Nanog* and *Gdf3* in murine ESCs, we next analyzed the expression of these factors in different iPS lines. The characteristics of these iPS cells are outlined in Supporting Information Figures S5–S7. We first tested the SmartFlare™ Probes on murine iPS cells derived from tail tip fibroblasts of the Nkx2.5 CE eGFP mouse line [26]. The iPS colonies were clearly stained with probes specific for *Nanog* and *Gdf3* (Fig. 3A). Likewise, SmartFlare™ Probes successfully detected expression of *NANOG* and *GDF3* in human (Fig. 3B) and porcine (Fig. 3C) iPS cells. The results of the immunocytochemical staining of NANOG protein coincided nicely with the results obtained by SmartFlare™ Probes in murine and human iPS (Fig. 3A, 3B). Furthermore, the strong expression of *NANOG* and *GDF3* could be confirmed by qRT-PCR analyses in iPS cells of all three species (Fig. 3D).

Therefore, these results clearly indicate that SmartFlare™ Probes provide a reliable tool to detect the expression of pluripotency genes such as *NANOG* and *GDF3* in iPS cells of different species with a common reporter sequence directly in live cells. In addition, the SmartFlare™ data on gene expression are in good agreement with those obtained by other independent methods.

Live Screening of Successfully Reprogrammed Murine TTFs to iPS Cells

Our previous convincing results prompted us to assess the power of SmartFlare™-induced fluorescence to reliably identify developing iPS colonies directly *in situ*. To that end murine TTFs were reprogrammed with lentiviruses containing the doxycycline inducible pHAGE2-Tet-STEMCCA [28] and a constitutively expressed reverse tetracyclin transactivator. The first iPS-like colonies were visible after 1 week. Nine days after lentiviral transduction *Nanog*- or *Gdf3*-specific SmartFlare™ Probes were added (Fig. 4A). On day 10, we screened the colonies microscopically for the appearance of Cy3 fluorescence. We obtained colonies with a rather faint fluorescence (FF colonies) while others displayed a very bright fluorescence (BF colonies). Representative live fluorescence images are shown in Figure 4B. The exposure time for the FF colonies had to be significantly increased to record the fluorescence of both *Nanog* ($p = .022$) and *Gdf3* ($p = .018$) (Fig. 4C). Based on the fluorescence intensity several FF and BF colonies screened by *Nanog*- ($n = 9$) or *Gdf3*-specific ($n = 10$) SmartFlare™ Probes were picked on day 11, expanded, and analyzed. Quantitative RT-PCR analysis of pluripotency markers at passage 2 after picking revealed significantly higher expression levels of *Oct4*, *Nanog*, and *Gdf3* in *Nanog*-SmartFlare™-selected BF colonies when compared with their FF counterparts (Fig. 4D). In addition, we saw a nice correlation between gene expression of *Nanog* at passage 2 and the light exposure time in those colonies screened after application of SmartFlare™ Probes specific for *Nanog* but not in those selected by *Gdf3* (Supporting Information Fig. S8). Immunocytochemical detection of Nanog protein in FF and BF colonies confirmed this result (Fig. 4E).

We then compared the capacity of *Nanog*-selected colonies to differentiate into cells of the three germ layers during an undirected *in vitro* differentiation assay by measuring the expression level of lineage-specific marker genes in 7-day-old embryoid bodies. Differentiating BF colonies strongly upregulated *Afp* (endoderm-specific), *Ncam1* (ectoderm-specific), and the two early cardiac mesodermal genes *Nkx2.5* and *Hand2* (Fig. 4F). In contrast, FF colonies almost completely failed to do so (Fig. 4F). Finally, we verified the frequency of cardiac precursor cells upon undirected differentiation which can easily be identified by the expression of GFP in cells derived from the Nkx2.5 CE eGFP transgenic mouse line [26]. Consistent with the gene expression results, the efficiency of generating cardiac precursor cells was more than threefold higher with BF compared to FF colonies (Fig. 4G).

Thus, we were able to perform a live screening for the appearance of truly reprogrammed iPS cells based on the SmartFlare™-induced intensity of fluorescence. Brighter clones expressing appropriate pluripotency markers were able to efficiently differentiate into cells of all three germ layers and also showed an increased capacity to develop into cardiac precursors.

Discussion

In this study, we have performed a novel RNA-based approach to detect intracellular gene expression directly in live cells by fluorescent SmartFlare™ Probes. Our results using several SmartFlare™ Probes targeting different genes in various cell lines demonstrate the suitability of the method for reliable detection of specific intracellular gene expression, allowing live imaging and FACS sorting of cells expressing the target gene, without any perturbation of its expression. This approach has many advantages compared to other methods. It allows the detection of gene expression directly in live cells without any restriction of the target gene or cell type and fluorescent signals are bright enough to be appreciated with a fluorescence microscope even in individual cells. In contrast

to the recently described fluorescent molecular beacons (dual-labeled antisense oligonucleotides with hairpin structures) that allow live staining after nucleofection of single cells in suspension [30], the SmartFlare™ Probes can simply be applied to the culture medium without any need for manipulation or selection of the cells as the particles are internalized by endocytosis. After the addition of SmartFlare™ Probes and recording of the fluorescence the gold nanoparticles exit the cells by exocytosis while fluorescent reporter probes are degraded by nuclease activity which allows further culture. Therefore, one does not lose any precious cells and there is no requirement for replicate cultures. We could successfully show this for murine iPS cells which were devoid of any fluorescent signal after their passage and they maintained a normal phenotype and growth properties for at least 5 days after the application of SmartFlare Probes (Supporting Information Fig. S9). Moreover, by selection of appropriate reporter sequences, which are not restricted to special secondary structures of the RNA as for molecular beacons [30], it is possible to use a single probe that detects gene expression across species. This worked successfully for all genes which we have investigated. Finally, the SmartFlare™ technology is quite powerful in cases when antibodies directed against the gene product are not available. Looking at the advantages, it appears negligible that certain markers of pluripotency such as SSEA1 and TRA-1–81 are not detectable by this method as the epitopes mapped by the antibodies represent carbohydrates [31].

We focused our work on pluripotent cells in which the detection of intracellular transcription factors is of particular interest to verify their pluripotency. Using the established and characterized murine Nkx2.5 CE eGFP and V6.5 ESC lines we could show the functionality of probes specific for *Nanog* and *Gdf3*. The *Nanog*-specific probes NANOG-SF1 and SF2 that have partially overlapping sequences were equally effective and both induced a strong fluorescence. As a second marker associated with undifferentiated ESCs we analyzed *Gdf3*, which is a secreted type 4 TGF-β ligand [32]. Both *Gdf3*-specific reporters clearly validated that the gene is expressed in Nkx2.5 CE eGFP and V6.5 ESCs.

The expression level of certain pluripotency markers required to maintain pluripotency might be quite low under certain circumstances [33]. Therefore, it would be deleterious if the addition of SmartFlare™ Probes impaired the expression level of the respective target gene. We have performed comparative qRT-PCR analyses and could show that the addition of SmartFlare™ Probes did not negatively influence the mRNA and protein level of *Nanog* or *Gdf3* in murine ESCs. Therefore, the application of SmartFlare™ Probes should be feasible without the fear to knock down mRNA or protein expression of any target gene.

The fact that SmartFlare™-treated live cells can subsequently be cultured and expanded prompted us to investigate the potential of SmartFlare™ Probes as a tool to screen for the appearance of successfully reprogrammed murine iPS cells. Indeed, we were capable to identify such colonies based on the intensity of the fluorescent signal after addition of *Nanog*- and *Gdf3*-specific probes. Characterization of single iPS clones after expansion demonstrated that brighter positive colonies were indeed pluripotent confirming the accuracy of our screening approach. Furthermore, the screening with *Nanog*-specific SmartFlare™ Probes revealed that the intensity of the fluorescence correlated with the performance of an individual clone in subsequent differentiation assays. Strong fluorescing BF colonies developed with an enhanced efficiency into cells of all three germ layers and produced three times more cardiac precursor cells than low fluorescing FF colonies. This is in good agreement with other reports where high expression of Nanog protein correlated positively with the capacity of the cells to effectively undergo differentiation [18].

The use of *Nanog* to identify truly reprogrammed pluripotent cells is a controversial issue. There are reports that suggest a fluctuating expression of *Nanog* in murine ESCs [34]. In contrast, other groups confirm bona fide human iPS cells by qRT-PCR for *NANOG* [18, 25] and this correlation held true in downstream experiments such as teratoma formation in immunodeficient mice [18]. We also have chosen *Nanog* as a marker to identify truly reprogrammed murine iPS cells in situ. Due to the elevated pluripotency gene expression and the improved differentiation capacity of BF colonies obtained after screening with *Nanog*-specific SmartFlare™ Probes we are quite confident that *Nanog* is a reliable selection marker in our approach to identify truly reprogrammed murine iPS cells.

In our screening approach, we have used individual genes and Cy3 as the only reporter fluorescence. However, as Cy5-tagged SmartFlare™ Probes are also available, it is imaginable to add further dyes and combine many fluorescent markers in one nanoparticle to obtain a "rainbow" SmartFlare™ Probe which enables the simultaneous detection of multiple pluripotency genes. Such a multifluorescence signal should highly strengthen the reliability to designate an individual colony as truly reprogrammed.

Upon cardiac differentiation Nkx2.5 CE eGFP ESCs allow the identification of cardiac precursor as a consequence of the activation of an *Nkx2.5* cardiac enhancer element [26]. With the SmartFlare™ technology, it should be possible to trace the expression of any desired gene over an extended time period by repeated addition of the appropriate probe. By such an approach the success of differentiation of ESCs or iPS cells into a certain lineage could be evaluated by a "real-time follow-up" of the expression of relevant marker genes.

The fluorescent labeling of intracellular gene expression opens the possibility to apply this technique in different research areas. Staining of living cells with a specific fluorescence would facilitate the enrichment and purification of populations positive for a specific marker by FACS. For our approach aimed at the qualitative detection of pluripotency gene expression the labeling efficiency achieved using nonsaturating concentrations of SmartFlare™ Probes was sufficient, as validated in downstream differentiation experiments. To select rare cell populations, however, it is desirable to label almost every potential target cell. Indeed, by raising the concentration of SmartFlare™ Probes the frequency of Cy3-positive cells growing either as monolayers (293 cells) or in three-dimensional structures (colonies of Nkx2.5 CE eGFP ESCs) can be increased tremendously (Supporting Information Fig. S10). Thus, by applying the appropriate concentration of SmartFlare™ Probes, which may vary in different cell types, the vast majority of a given target population should be labeled. For example, a cardiac precursor population positive for *Islet1* has recently been described [35–37]. This rare population could be enriched by FACS sorting from iPS cultures using an *Islet1*-specific SmartFlare™ Probe. Subsequently,

these cells could be cultured, expanded, and characterized in detail. In addition, the successful enrichment of such a population should have an enormous potential in subsequent (xeno)transplantation experiments, as the cells retain the unique characteristics of an unaffected and unaltered genetic background. Recently, SmartFlare™ Probes have been successfully used to selectively stain live ventricular myocytes [38], melanoma subpopulations [39], and *POP3* expressing monocytes [40] underlining the general potential of this method to identify any cell population defined by a specific marker gene.

Efforts are undertaken to establish large iPS banks as a repository for research in drug screening, disease modeling, and regenerative cell-based therapies [41]. The establishment of such biobanks requires high-throughput technologies which enable the generation of such lines and which guarantee the application of appropriate quality controls. In addition, time is an essential factor in a potential cardiac therapy using iPS cells which demands automated processes where possible. The identification of truly reprogrammed iPS cells has been achieved by reference expression scorecards [23] or cells were purified by flow cytometry based on surface markers [25]. Our approach with the SmartFlare™ technology allows both approaches: marker gene expression can be detected by automated fluorescence microscopy and combined with an appropriate robotic sampling system or a subsequent purification by flow cytometry.

Various genome editing tools such as zinc fingers, TALENs, and CRISPR nucleases [42] have been developed to genetically engineer and correct genetic defects in patient-derived iPS cells. The gene transfer may correct disease-causing point mutations [43] or rescue the phenotype by providing a deficient gene into the safe harbor AAVS1 locus [44]. This approach can be supported by SmartFlare™ Probes in two ways: first, they represent an easy tool to verify the pluripotency status of the iPS cells after the manipulation to identify the most appropriate colonies. Second, they can be applied as an optical tool for downstream screening for the functional restoration of the defect without limitation of the target gene or cell type.

Summary

In summary, we have established a novel approach that allows the detection of gene expression directly in live cells without any restriction of the target gene or cell type across species borders using appropriate oligonucleotide reporter sequences. In the area of stem cell research, the application of SmartFlare™ Probes allows the detection of pluripotency genes, which so far were traceable only after fixation, thus improving, facilitating, and accelerating the identification of fully reprogrammed colonies. In addition, this approach is a very powerful tool applicable to all research areas for the detection and the specific selection of populations characterized by intracellular factors. Finally, the SmartFlare™ Probes may be valuable tools to further improve the efficiency of high-throughput and genome editing technologies.

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Author Contributions

H.L. and S.D.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript; M.D., D.S., K.A., A.W., T.B., M.-A.D., and M.S: collection and/or assembly of data and final approval of the manuscript; K.-L.L., R.L., A.M., and M.K.: conception and design, financial support, data analysis and interpretation, and final approval of manuscript. H.L., S.D., A.M., and M.K. contributed equally to this article.

Disclosure of Potential Conflicts of Interest

H.L. has received the SmartFlare™ Probes from Merck Millipore free of charge. All other authors declare no conflict of interest.

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