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# Real-time and Non-invasive Monitoring of Embryonic Stem Cell Survival during the Development of Embryoid Bodies with Smart Nanosensor

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**Abstract**

Embryonic stem cells (ESCs)-derived embryoid body (EB) is a powerful model for the study of early embryonic development and the discovery of therapeutics for tissue regeneration. This article reports a smart nanosensor platform for labeling and tracking the survival and distribution of ESCs during the EB development in a real-time and non-invasive way. Compared with the cell tracker (i.e. DiO) and the green fluorescent protein (GFP), nanosensors provide the homogenous and highly-efficient ESC labeling. Following the internalization, intracellular nanosensors gradually release the non-fluorescent molecules that become fluorescent only in viable cells. This allows a continuous monitoring of ESC survival and distribution during the process of EB formation. Finally, we confirm that nanosensor labeling does not cause the significant influences to biological properties of the ESCs and EBs.

**Key words:** embryonic stem cell, embryoid body, viability, nanosensor, real-time and non-invasive monitoring

**Statement of Significance**

The distribution pattern of viable embryonic stem cells (ESCs) within embryoid body (EB) is closely related with the maturation of EBs. Noninvasive and real-time monitoring of viable ESC distribution in EBs would allow researchers to optimize the culturing condition in time during the EB development and to select the suitable EBs for subsequent applications.

## 1. Introduction

Embryonic stem cells (ESCs) are a population of self-renewing, pluripotent cells that are able to differentiate into cell types representing all three germ layers [1, 2]. ESCs-derived embryoid bodies (EBs, spherical aggregates of ESCs in suspension) recapitulate critical events during early embryo development, which offers a perfect *in vitro* platform to study cell localization, distribution, viability and lineage commitment during embryonic development [3, 4].

The distribution pattern of viable ESCs within EBs is closely related with the maturation of EBs. With the progress of ESC differentiation, programmed cell death (apoptosis) gradually occurs in the core of EBs [5, 6]. Additionally, several growth factors commonly used to promote

ESC differentiation, such as bone morphogenetic protein (BMP) [7-9] and fibroblast growth factor (FGF) [10, 11], are also reported to be involved in inducing cell apoptosis in EBs. Therefore, noninvasive and real-time monitoring of ESC viability distribution in EBs would allow researchers to optimize the culturing condition in time during EB development and to select the suitable EBs for subsequent applications.

To monitor the *in vitro* cell viability continuously and non-invasively, fluorescent labels are always preferred due to the low-cost and convenience. For example, lipophilic carbocyanine dyes (e.g. DiO) can selectively label the plasma membrane [12] and has been utilized for the visualization of cell migration and proliferation [13-16]. Unfortunately, carbocyanine dyes cannot provide any information about the cell properties like viability. Another approach is to introduce fluorescent reporter genes (e.g. sequences coding green fluorescent protein (GFP)) into cells of interest [17]. Only if the cells are viable, there are the fluorescent proteins that can be used as the markers for tracking the cell migration and survival [18, 19]. For example, transplantation of GFP-expressing tumor cells into nude mice allowed tumor cell invasion and metastasis to be visualized *in vivo* for the first time [20, 21]. The only problem about the reporter gene-based strategy is the low non-viral transfection efficiency in primary cells and stem cells (usually <10%) [22, 23]. In addition, there might be mutations in genetically modified cells as a result of exogenous gene insertion [24, 25].

Previously, we have designed a nanoparticle-based sensor platform to track real-time expression of specific biomarkers that correlate with cell status and functions [26, 27]. Based on this platform, we developed a viability nanosensor by incorporating non-fluorescent calcein acetomethoxy (CAM) into poly (lactic-co-glycolic acid) (PLGA) nanoparticles [28]. When internalized by cells, PLGA nanoparticles gradually degrade within the cytoplasm and continuously release encapsulated CAM, which are then converted by esterases in living cells into fluorescent calcein molecules. As a result, CAM nansensors can be used not only for cell labeling but also for monitoring cell viability.

Here, we report the use of viability nanosensors for highly-efficient ESC labeling and non-invasive monitoring of ESC survival and distribution during EB development (Fig. 1). As comparisons, lipophilic carbocyanine dye (i.e. DiO) and GFP reporter gene are used in parallel. Following the homogenous and highly-efficient internalization, intracellular nanosensors

gradually release the non-fluorescent molecules that become fluorescent only in viable cells. While signals from these nanosensors show a steady decrease from day 1 to day 7 due to the rapid cell division within EBs, the unique cell viability monitoring function of nanosensors allows a clear presentation of living cell distribution within the EBs. The analysis of ESC proliferation rate, pluripotency maintenance and differentiation capacity are used for proving the biosafety of the nanosensors.

## 2. Materials and Methods

All chemicals except mentioned specifically were all purchased from Sigma-Aldrich and used without further purification. All cell culture reagents and supplements were obtained from Life Technologies (USA), unless otherwise stated.

***2.1 Viability nanosensor synthesis*:** 100 mg of PLGA (lactide:glycolide = 50:50)) was dissolved in 2 mL chloroform at 4 $^{o}$C and then mixed with 250 µg of CAM (Life technologies) in 250 µL dimethyl sulfoxide (DMSO). The mixture was added dropwise into 3% (w/v) poly (vinyl alcohol) (PVA) solution followed by homogenization (Tissue Master 125, Omni International) at 24,000 rpm for 1 min. The resulted emulsion was then placed in chemical hood overnight for the evaporation of chloroform. Finally, nanosensors were collected by centrifuging at 6,000 rpm for 5 minutes, washed thrice with double-distilled water, and freeze-dried (-80 $^{o}$C) before being stored in -20 $^{o}$C prior to usage.

***2.2. Murine embryonic stem cell (mESC) culture*:** mESC (E14TG2a, American Type Culture Collection (ATCC)) were maintained on tissue culture flasks (Falcon) pre-coated with 0.1% (w/v) gelatin. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (FBS, PAA Laboratories), 0.1 mM nonessential amino acids (NEAA, Gibco), 0.1 mM 2-Mercaptoethanol (2-ME) and 1000 units/mL leukemia inhibitory factor (LIF, Millipore). Upon reaching 80% confluence, the cells were trypsinized and used for subsequent experiment. Only cells with passage number of 6 to 10 were used for experiments in this study.

***2.3 Cell labeling with nanosnesors*:** 1 mg nanosensors were placed in 100 μL of 0.1% (w/v) poly-L-lysine (PLL) aqueous solution for approximately 20 minutes. Then, PLL-coated nanoparticles were purified through centrifugation and re-dispersed in 1ml mESC culture medium. mESCs were plated on the gelatin-coated flask overnight before being incubated with nanosensor-containing medium. 12 hours later, the nanosensor-containing medium was then replaced with normal mESC culture medium.

***2.4 Cell labeling with DiO*:** 2 μL DiO cell-labeling solution was added to 1 ml mESC culture medium to prepare the 0.2% (v/v) working solution. Then mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution for 4 hours. The DiO-containing medium was then replaced with normal mESC culture medium.

***2.5 Cell labeling with enhanced GFP (EGFP) plasmid*:** 400 ng pEAK12-EGFP plasmid (promoter: cytomegalovirus, Clontech, USA) was mixed with 1 μL Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 20 minutes before being added to 500 μL mESC culture medium to prepare the working solution. mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution. 6 hours later, the Lipofectamine-containing medium was replaced with normal mESC culture medium.

***2.6 Fluorescence imaging*:** All fluorescent images were captured with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Then fluorescent positive population percentage and average cellular fluorescent intensity were calculated. First, fluorescent area and average fluorescent intensity in one fluorescent image were quantified with ImageJ. Cell-covered area in according phase contrast image was also calculated with ImageJ. Then fluorescent positive population percentage and average cellular fluorescent intensity were achieved by normalizing the fluorescent area and average fluorescent intensity to the cell-covered area, respectively. For each group, at least 6 images of each group were captured and analyzed.

***2.7 Flow cytometry analysis.*** Cells were dissociated from culture plate using 0.25% trypsin (Gibco) and fixed in 4% (w/v) paraformaldehyde at room temperature for 20 minutes. After being

washed with PBS thrice, 1 million cells from each group were re-suspended in 0.5% (w/v) bovine serum albumin (BSA) solution. Flow cytometry analysis was performed on LSRFortessa X-20 (BD Biosciences) and analyzed with the BD FACSDiva Software (BD Biosciences) and FlowJo (Tree Star).

***2.8 Cell proliferation assay*:** mESCs were seeded at a density of $5.0 \times 10^4$ cells/well onto a 24-well tissue culture plate and labeled as above mentioned. Following labeling, the cells were continuously cultured for 7 days. At various time points (day 1, 3 and 7), the culture medium was removed, and the cells were incubated with 500 µL culture medium supplemented with 50 µL of WST-1 reagent (Roche) for 3 hours. The supernatant was then collected and examined (absorbance: 450 nm; reference: 620 nm) with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). To obtain proliferation rate, readings at different time points were normalized against that of day 1 for each group. At least three replicates (n=3) from each group were assessed.

***2.9 EB formation assay and ESC three-germ layer differentiation*:** To initiate EB formation, mESCs were dissociated into single cells using 0.25% trypsin (Gibico) and cell suspension droplets consisting of 1000 cells in 20 µL of differentiation medium were hung on the lids of petri dishes to make "hanging drops" [29]. The differentiation medium composed of Iscove's modified Dulbecco's medium (Gibco) supplemented with 15% FBS, 0.1 mM NEAA and 0.1 mM 2-ME. 48 hours later, the EBs were transferred onto non-adherent petri dishes. For EB formation assay, EBs cultured in suspension conditions for another 5 days. For ESC three germ layer differentiation, EB cultured in suspension conditions for 3 days and were seeded onto 0.1% gelatin-coated 24-well tissue culture plate on day 5. From day 5 to day 14, differentiation medium was changed every other day.

***2.10 Confocal imaging*:** EBs were fixed in 4% (w/v) paraformaldehyde solution at $4^oC$ and washed thrice with PBS before imaging. Samples were imaged with confocal microscope LSM710 (Zeiss). Florescence intensity distribution across EBs developed from ESCs labeled with DiO or nanosensor on day 7 was also analyzed with the ImageJ software. The center of EB was

designated as ‘0’ and used as reference to normalize all other positions.

***2.11 Gene expression analysis*.** Gene expression analysis was performed by using quantitative real-time polymerase chain reaction (qPCR). At various time points, mESCs or EBs were dissolved in TRIzol (Gibco, ThermoScientific) for extraction of RNA, which was then converted into cDNA via reverse transcription prior to gene expression evaluation through qPCR. All the reverse transcription reagents used were obtained from Promega (USA). Subsequently, qPCR was done using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Singapore) and the CFX Connect Real-Time System (Bio-Rad, Singapore). Gene markers analyzed in this study include OCT4 and Nanog for stem cell pluripotency; Sox17 and GATA4 for endoderm differentiation; Brachyury (T) and PDGFRα for mesoderm differentiation; Sox1 and OTX2 for ectoderm differentiation. Table 1 listed primers (AIT Biotech, Singapore) used in this experiment. For analysis, target gene expression was first normalized to housekeeping gene β-actin in the corresponding sample. Then the relative target gene expression was further normalized to the relative target gene expression in day 1 sample from positive control group. Three replicates from each different modified PDMS substrate (n=3) were used to determine each target gene expression.

***2.12 Immunohistochemistry staining*:** mESCs or EBs were fixed in 4% (w/v) paraformaldehyde at 4$^{o}$C until staining. Samples were washed thrice with PBS prior to immunostaining. Nanog antibody (rabbit polyclonal IgG, sc-33760, Santa Cruz Biotechnology) and OCT4 antibody (goat polyclonal IgG, sc-8629, Santa Cruz Biotechnology) were used for pluripotency evaluation. Sox-17 antibody (goat polyclonal IgG, sc-17356, Santa Cruz Biotechnology) and GATA4 antibody (goat polyclonal IgG, sc-1237, Santa Cruz Biotechnology) were used for endoderm differentiation assessment. Brachyury antibody (goat polyclonal IgG, AF2085, R&D Systems) and PDGFRα antibody (goat polyclonal IgG, AF1062, R&D Systems) were used for mesoderm differentiation assessment. OTX2 antibody (goat polyclonal IgG, sc-30659, Santa Cruz Biotechnology) and Sox-1 antibody (goat polyclonal IgG, sc-17318, Santa Cruz Biotechnology) were used for ectoderm differentiation assessment. Samples were incubated with according primary antibody diluted at 1:200 in 10% (v/v) horse serum and 0.4% (v/v) Triton X-100 at 4 ℃ for overnight. After

that, samples were washed with PBS and incubated with Goat anti-Rabbit IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-11010, Invitrogen) or Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-21085, Invitrogen) diluted at 1:200 in 10% (v/v) horse serum and 0.4% (v/v) Triton X-100 at room temperature for 1 hour in the dark. Nuclei were counterstained by 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Stained samples were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus).

***2.13 Statistical Analysis*:** All results were presented as mean ± standard deviation. One-way ANOVA was used for comparisons assuming equal variances followed by least significant difference (LSD) test. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1 ESC labeling using nanosensors

The nanosensors were synthesized according to our previous studies [28]. Nanosensors used in this study was spherical in shape and ~ 1 μm in size (Fig. S1a). Right after synthesis, nanosensor was almost neutral (-0.1 mV). However, PLL coating resulted in positively charged nanosensors (+15.5mV), which assists in their adsorption to cell membrane and subsequently internalization. When incubated in cell culture medium with 10% FBS at 37 °C, only 10% of encapsulated CAM was released over the first 24 hours (Fig. S1b). In addition, when placed in cell culture medium with 10% FBS at 37 °C, the average size of nanosensor only increased by 10% after 24 hours of serum exposure and 20% after 96 hours (Fig. S1c). These results showed good stability of the nanosensor during serum exposure, appropriate for cell labeling which occurs on the first 24-hour.

The ESCs were labeled with nanosensors through the simple incubation. One key parameter in this step was the concentration of nanosensors. We screened 5 concentrations (0.1, 0.5, 1, 2, 4mg/mL) and found out that a higher concentration of nanosensors resulted in a high cellular fluorescence (Fig. S2). However, we observed significant loss of cell viability when labeling concentration exceeds 1 mg/mL (Fig.S3). The low cellular fluorescence in 4mg/mL labeling group is also likely a result of poor cell viability. Therefore, to achieve a balance between labeling efficiency and cell viability, ESCs were labeled with 1 mg/mL nanosensors for all the following

experiments. The nanosensors at this concentration showed comparatively-strong signal intensity while the minor effect on ESC viability.

As comparisons, ESCs were also labeled with DiO, a lipophilic carbocyanine dye that inserts into cell membrane and GFP plasmid transfection through Lipofectamine. As shown in Fig. 2a, nanosensors showed the highest labeling efficiency, while there were only a few fluorescent cells for GFP plasmid transfection group. Flow cytometry analysis (Fig. 2b) revealed that nanosensors labeled 99.8% cells. Meanwhile, DiO labeled 92% cells and only 7.52% cells expressed GFP in the plasmid transfection group. Thus, nanosensor labeling outperformed other two methods in terms of labeling efficiency.

**3.2 Dilution of nanosensors in ESCs upon cell divisions**

One challenge for labeling cells with nanoparticles is the dilution of intracellular nanoparticles, which is due to the exocytosis of nanoparticles and division of cells [30]. To study the signal dilution of nanosenors in ESCs, labeled ESCs were continuously observed for 7 days, since EB period in routine ESC differentiation protocols usually lasts 5-7 days [31].

As showed in Fig. 3a, fluorescent signals in nanosensor-labeled ESCs decreased significantly from day 1 to day 3, but subsequently remained relatively constant until day 7. Signal dilution was also observed in DiO group, though the attenuation was less compared to the nanosensor group. On the contrary, the signal of GFP-expressing cells increased gradually over the 7 days of culture, most likely due to the accumulation of GFP within individual cells. Clearly, if it were not for the risk of mutagenesis and extremely low transfection efficiency of non-viral transfection, the GFP-labeling would be the best choice for tracking ESCs in the EB development.

Fluorescent signal dilution was further analyzed by quantifying fluorescent-positive population and average cellular fluorescent intensity of each group (Fig. 3b and c). Both DiO and nansensor groups showed a reduction in fluorescent-positive population. Nanosensor group had 85.21% fluorescent-positive population on day 1, 75.61% fluorescent-positive population on day 3, and 42.16% fluorescent-positive population on day 7. DiO group showed lower fluorescent-positive population, with 59.18% fluorescent-positive population on day 1, 51.52% fluorescent-positive population on day 3, and 46.37% fluorescent-positive population on day 7. In contrast, GFP had a constant but quite low fluorescent-positive population, with 0.69%

fluorescent-positive population on day 1, 1.86% fluorescent-positive population on day 3, and 0.63% fluorescent-positive population on day 7 (Fig. 3b).

Average cellular florescence intensity also decreased in both nansensor and DiO groups. Nanosensor group showed 41.94 cellular florescence intensity on day 1, 14.38 cellular florescence intensity on day 3, and 9.48 cellular florescence intensity on day 7. DiO group showed initial low while steadily decreased cellular florescence intensity, with 23.82 cellular florescence intensity on day 1, 16.47 cellular florescence intensity on day 3, and 14.37 cellular florescence intensity on day 7. Though quite low in cellular florescence intensity, GFP group showed an increasing and subsequently decreasing signals intensity, with with 0.24 cellular florescence intensity on day 1, 0.97 cellular florescence intensity on day 3, and 0.27 cellular florescence intensity on day 7 (Fig. 3c).

**3.3 Influence of nanosensors on ESC proliferation and pluripotency maintenance**

Besides the dilution of nanosensors, another concern is the potential influence of nanosensor labeling on the proliferation and pluripotent capacity of ESCs. To test the influence of nanosensors on ESC proliferation, WST assay was performed at various time points (day 1, 3 and 7) after labeling. As shown in Fig. S4a, nanosensors and DiO didn't cause any influence over the cell proliferation rate. However, GFP labeling with Lipofectamine transfection generated a significant difference when compared to unlabeled control group on day 7. This might be due to lower starting cell density due to loss of viability following Lipofectamine transfection. In consequence, remaining viable cells had more access to nutrients and proliferated faster.

Next, we examined the pluripotency capacity of ESCs after nanosensor labeling through gene expression analysis and immunostaining. OCT4 and Nanog are commonly-used pluripotent biomarkers for ESCs. Fig. S4b and c revealed that both of these two gene markers exhibited an up-regulation trend in nanosensor group along the culture period, with a slight difference as compared to control group. Meanwhile, DiO and GFP labeling also showed similar impact on OCT4 and Nanog gene expression. These results indicate that labeling minimally affected differentiation potential of ESCs. Moreover, immunostaining for both OCT4 and Nanog also confirmed the result of gene expression study (Fig. S4d). All groups manifested positive staining for OCT4 and Nanog (red color) at the end of 7-day *in vitro* culture, even though slightly stronger

staining was observed in control group.

### 3.4 The development of EBs with nanosensor-labeled ESCs

EBs are cell aggregates which are formed due to ECSs proliferation and differentiation in suspension condition. During the development of EBs, ESCs labeled with nanosensors, DiO or GFP were suspended in differentiation medium and then hung on the lids of petri dish to make "hanging drops". EBs were well-formed 24 hours after hanging drop fabrication.

The formed EBs were maintained in culture medium and imaged for 7 days (Fig. 4a). On day 1, both nanosensor and DiO EB groups showed a strong and homogenous fluorescent signals through the whole cell aggregates. In contrast, the fluorescent signal in GFP EB group had an imbalance distribution.

As EBs developed from day 2 to day 7, all three groups lost signals gradually (Fig. 4b). The signal attenuation of DiO was the slowest and the fluorescence was still pretty strong on day 7 (Fig. 4b and S5). Nanosensor group lost the signal in a faster way (Fig. 4b and S6). The performance of GFP group was the worst and the fluorescent signal was completely lost on day 7 (Fig. 4b and S7).

One thing to note is the dark core of EBs in the phase contrast images in all three groups on day 5 and day 7 (Fig. 4a), which were necrotic center developed in EBs because of gradually-increasing EB size beyond nutrition diffusion limitation. As CAM released from nanosensors could only label living cells, we then evaluated whether the observed signal decrease was due to the loss of cell viability in the necrotic core of EBs.

### 3.5 Cell viability reporting function of nanosensors during EB formation

As the results showed in live/dead staining of EBs developed from unlabeled ESCs (Fig.S8), the whole EBs were stained green (indicative of live cells) with rare red-stained cells (indicative of dead cells) on day 3. Along with EB development from day 5 to day 7, cells at the edge of EBs still was stained positively for green. However, cells in the core of EBs were stained red. This suggests that cells in the core of EBs gradually die and form necrotic cores in EBs.

The EBs developed from labeled ESCs were then closely examined with confocal microscope (Fig. 5a). On day 5 and day 7, positive signals were still observed on the edge of the

nanosensor-labeled EBs, with the center region showing no fluorescence (thereby forming a negative core within a positive shell). In contrast, DiO group showed homogenously-distributed signals across the whole EB (a positive core within a positive shell) even until day 7. In GFP group, signals disappeared from day 5 to day 7, leaving completely-dark areas in the field. As a result, EBs with a negative core and a negative shell were formed.

Through florescence intensity quantification of EB cross-section images, the difference in signal distribution between the nanosensor and DiO group was further revealed. As revealed in Fig.5b, the florescence signal on two edges of EBs was clearly much stronger than that in the center in the nanosensor group. In DiO group, there was no significant difference between the edge and center of EBs. These results indicate that the decrease in signal intensity in EBs from the nanosensor group was partially due to the presence of non-fluorescenct dead cells that accumulated in the core of EBs. These results also demonstrate the unique live/dead reporting function of viability nanosensor during the process of EB formation.

**3.6 The effects of nanosensor labeling on ESC differentiation**

Finally, the effects of nanosensor labeling on ESC three-germ layer differentiation were examined via qPCR and immunostaining. Two representative genes for each germ layer were analyzed in order to more accurately evaluate differentiation status of ESCs. Sox17 and GATA4 genes were used as endoderm biomarkers; Brachyury (T) and PDGFRα genes as mesoderm biomarkers; and Sox1 and OTX2 genes as ectoderm biomarkers. Fig. 6 revealed that nanosensor and DiO groups expressed three-germ layers-associated genes in an increasing trend, whereas GFP group exhibited a downregulated trend along the culture period. While there were differences in the extent to which these genes were expressed between labeled and unlabeled ESCs, the protein biomarkers could be still positively stained in all the three labeling groups (red color), with nanosensor and DiO group showing more intensive color than the GFP group (Fig. 7). Only a slight difference in staining degree could be found between the nanosensor group and the control group. These data demonstrate that three-germ layers can be successfully specified in nanosensor-labeled ESCs and that nanosensor only has minor inhibitory effects on ESC three-germ layer differentiation.

## 4. Discussion

This study explores the utilization of viability nanosensors to label ESCs and monitor their survival during the development of EBs. These nanosensors were made through encapsulating CAM (a widely-used dye for labeling viable cells) within PLGA nanoparticles. Once internalized by the ESCs, PLGA started to degrade and CAM molecules were slowly released. Under presence of esterases, the non-fluorescent CAM was converted to the strong fluorescent calcein. As the enzymes were only present in the viable cells, the fluorescent signal could reflect the viability of cells. In comparison to GFP transfection with Lipofectamine, nanosensor labeling exhibited a high labeling efficiency (Fig.2). The efficiency of GFP transfection for ESCs in this study was less than 10%, which was consistent with previous reports [32-36]. In comparison to DiO that is known to label cells in non-uniform manner [37], the distribution of fluorescent signals in the nanosensor group was more homogenous. These results imply the feasibility of using CAM nanosensors as a powerful tool to label ESCs.

The dilution of nansensor signals in ESCs was found to be quick in this study, with 10% decrease in fluorescence-positive population and 50% loss of cellular fluorescent intensity from day 1 to day 3 (Fig. 3). In our previous study, we showed that stable CAM signal could be observed from nanosensor-labeled MSCs for at least few weeks, better retained than DiO labeling [28]. The difference was probably due to the much more rapid proliferation rate of ESCs as compared to MSCs [38]. Even though the signal decrease in nanosensor group was more obvious than DiO group (Fig. 3c), the fluorescence-positive population in nanosensor group was higher than DiO group (Fig. 3b). PLGA nanoparticles can serve as a reservoir for continuous CAM release, which might be responsible for the higher fluorescence-positive population in nanosensor group [28]. For DiO group, cell division also led to a decrease in fluorescence-positive population and signal intensity (Fig. 3), as labeled plasma membrane of mother cells was allocated to two daughter cells upon division. Therefore, its retention is also quite poor in the rapidly growing ESCs. In comparison, GFP-transfected cells were mainly well-adhered (differentiated) cells with limited proliferation capacity. As a result, fluorescence-positive population and cellular fluorescent intensity increased from day to day 3 with the accumulation of GFP, even though both fluorescence-positive population and cellular fluorescent intensity were quite low in GFP group (Fig. 3b and 3c).

With the progress of differentiation, the cells in the core of EBs undergo programmed cell death and cavitation [6], which resembles the amniotic cavity formation during early embryo development in vivo [9, 39]. The size of EBs extending nutrition and oxygen diffusion limit is chiefly responsible for cell death occurring within the core of EBs [5, 40]. The presence of nanosensors in the ESCs allowed us to monitor the viability of cells in this situation in real time. As shown in Fig.5, cells in the periphery of EBs remained to be fluorescent, whereas the core of EBs lost their fluorescence, thereby forming a spheroid structure with fluorescent shell and dark core. This is consistent with the previous reports showing the occurrence of apoptotic cell death in the developed EBs [41, 42]. In contrast, there was fluorescent signal throughout the EBs formed by DiO-labeled ESCs. Although GFP-expressing ESCs in the GFP EB group presented undiluted fluorescence, both low transfection efficiency due to Lipofectamine as transfection reagent and possible transgene silencing [43, 44] resulted in the low percentage of cells expressing GFP, which hinders the visualization of all viable cells in EBs. Thus, the unique cell viability monitor function of viability nanosensors is well fulfilled in the EB formation model.

The analysis of ESC proliferation rate, pluripotency maintenance and differentiation capacity in this study proved the safety of the nanosensors. Nanosensor labeling did not affect ESC proliferation and only have minor inhibitory effects on cell pluripotency (Fig. S4). Meanwhile, for ESC differentiation potential, nanosensor labeling also did not cause severe inhibitions, with the presence of all three-germ layer biomarkers at the end of the differentiation experiment (Fig. 7). During the fabrication of CAM nanosensor, CAM was dissolved in DMSO prior to encapsulation. While DMSO is shown to induce ESC differentiation into multiple lineages [45, 46] and affect EB phenotypes [47], the slow degradation of nanosensors meant that we only observe minimal influences over these attributes due to nanosensor labeling.

## 5. Conclusion

In this study, we verified the feasibility of using viability nanosensors to label and track ESC status during EB development. Compared to DiO labeling and GFP plasmid transfection, nanosensor labeling showed homogenous and highly-efficient labeling. In particular, the unique cell viability monitoring function of nanosensors allowed clear presentation of live cell distribution within EBs. Therefore, viability nanosensors have high potential to be a powerful tool

to facilitate optimization of EB culturing condition and eventual selection. Future improvements focusing on enhanced signal stability and negligible inhibitory effects of the nanosensor would allow further application on actual growing embryos.

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**Figure Legends**

**Fig. 1.** Schematic illustration of nanosensor labeling and monitoring ESCs during EB formation.

**Fig.2.** Labeling efficiency of nanosensors on ESCs. (a) Florescent images of ESCs labeled by nanosensors, DiO and GFP transfection 24 hours later. (b) Flow cytometry analysis of labeled cells in nanosensor group, DiO group and GFP group. Green color indicates labeled cells.

**Fig.3.** Tracking of nanosensor-labeled ESCs for 7 days *in vitro*. (a) Florescent images of ESCs labeled by nanosensors, DiO and GFP transfection from day 1 to day 7. (b) Fluorescent-positive population in nanosensor group, DiO group and GFP group from day 1 to day 7. (c) Fluorescent intensity in nanosensor group, DiO group and GFP group from day 1 to day 7. Green color indicates labeled cells; The data are presented as the mean ± standard deviation (n=6). ** indicates $p < 0.01$.

**Fig.4.** The development of EBs with nanosensor-, DiO-, or GFP-labeled ESCs from day 1 to day 7. Green color indicates labeled cells; insets indicate phase contrast images of EBs.

**Fig.5.** Cell viability reporting function of nanosensors. (a) Confocal images of EBs developed from nanosensor-, DiO-, or GFP-labeled ESCs from day 3 to day 7. (b) Florescence intensity distribution across EBs developed from ESCs labeled with DiO or nanosensors on day 7. Green color indicates labeled cells.

**Fig.6.** Gene expression analysis of three-germ layer differentiation markers in nanosensor group, DiO group and GFP group from day 1 to day 7. The data are presented as the mean ± standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

**Fig.7.** Immunostaining for three-germ layer differentiation markers in nanosensor group, DiO group and GFP group 14 days after differentiation *in vitro*. Red color indicates positive staining; Blue color indicates cell nuclei.

# Supplementary Material

**Supplementary Methods**

***Dynamic light scattering (DLS):*** The hydrodynamic diameter of nanosensors was quantified with

Zetasizer nano Z (Malvern). Briefly, 1 mg of nanosensors were dispersed in 1 ml double-distilled water for Zetasizer measurement. For each sample, at least three measurements were performed.

***Scanning electron microscopy (SEM):*** Lyophilized nanosensors were plasma-coated with gold for 180 seconds and then imaged on JSM-6700F field emission scanning electron microscope (FESEM, JEOL; 5 kV).

***CAM release profile:*** To evaluate the release of CAM under serum condition, nanosensors were incubated in cell culture medium containing 10% FBS at 37 °C for 20 days. At designated time points (0.5, 1, 2, 4, 6, 8, 10, 12, 15 and 20 days), the supernatant was collected and treated with esterases (5U/ml). Following this, the fluorescence from the collected supernatant was read with Genios FL plate reader (TECAN). CAM concentration was then quantified by normalizing fluorescence readings against a standard curve generated from known quantities of CAM. Percentage values are obtained by normalizing the released quantities against the original loaded quantity.

***Serum stability:*** CAM nanosensors and blank nanosensors were incubated in cell culture medium containing 10% FBS at 37 °C for 10 days. On day 0, 1, 4,7 and 10, the hydrodynamic diameter of nanosensors was quantified with Zetasizer as previously described. For each sample, at least three measurements were performed.

***Live/dead staining:*** Qualitative live/dead fluorescent staining was performed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen) for EBs on day 3, 5 and 7. Briefly, calcein acetomethoxy (0.5 μL) and ethidium homodimer-1 (2 μL) were added to 1.0 mL of PBS to create staining solution. EBs were incubated in the staining solution for 30 minutes. Following this, EBs were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus).

**Supplementary Figures**

**Fig.S1.** Nanosensor characterization. (a) Hydrodynamic diameter of nanosensors and the

corresponding SEM image (inlet). (b) Release profile of CAM from nanosensors in cell culture medium with 10% FBS at 37 ℃. (c) Serum stability of CAM nanosensor and blank nanosensors in cell culture medium containing 10% FBS at 37 ℃.

**Fig.S2.** ESC labeling with various concentrations of nanosensors. (a) Florescent images of ESCs 24 hours after labeling with 0.1 mg/ml, 0.5 mg/ml, 1mg/ml, 2mg/ml and 4mg/ml nanosensors. (b) Fluorescent intensity of ESCs labeled with various concentrations of nanosensors. Green color indicates labeled cells; The data are presented as the mean ± standard deviation (n=6). ** indicates $p < 0.01$.

**Fig.S3.** Viability of ESCs 7 days after nanosensor labeling. The data are presented as the mean ± standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

**Fig.S4.** The influence of nanosensor labeling on ESC proliferation and pluripotency maintenance. (a) Cell proliferation assay for ESCs in nanosensor group, DiO group and GFP group from day 1 to day 7. Gene expression analysis of pluripotency markers OCT4 (b) and Nanog (c) in nanosensors group, DiO group and GFP group from day 1 to day 7. (d) Immunostaining of OCT4 and Nanog in nanosensors group, DiO group, GFP group and control group 7 days after labeling. Red color indicates positive staining; Blue color indicates cell nuclei; The data are presented as the mean ± standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

**Fig.S5.** Longitudinal assessment of EBs formed from DiO-labeled ESCs (left: green fluorescence channel; middle: phase contrast; right: merged channel).

**Fig.S6.** Longitudinal assessment of EBs formed from nanosensor-labeled ESCs (left: green fluorescence channel; middle: phase contrast; right: merged channel).

**Fig.S7.** Longitudinal assessment of EBs formed from GFP plasmid-transfected ESCs (left: green fluorescence channel; middle: phase contrast; right: merged channel).

**Fig.S8.** Live/dead staining of EBs developed from unlabeled ESCs from day 3 to day 7. Green color indicates live cells; red color indicates dead cells.