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Cell death, non-invasively assessed by intrinsic fluorescence intensity of NADH, is a predictive indicator of functional differentiation of embryonic stem cells

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Abstract

Background information—Continued advances in stem cell biology and stem cell transplantation rely on non-invasive biomarkers to characterise cells and stem cell aggregates. The non-invasive quality of such biomarkers is essential because exogenous labels, probes or reporters can unintentionally and dramatically alter stem cell state as can disruption of cell-cell and cell-matrix interactions. Here, we investigate the utility of the autofluorescent metabolite, nicotinamide adenine dinucleotide (NADH), as a non-invasive, intrinsic biomarker of cell death when detected with multi-photon optical-based approaches. To test this possibility, cell death was induced in murine embryoid bodies (EBs) at an early stage (day 3) of differentiation using staurosporine, an ATP-competitive kinase inhibitor of electron transport. Several hours after staurosporine treatment, EBs were stained with a single-colour, live/dead probe. A single-cross-sectional plane of each EB was imaged to detect the fluorescence intensity of the live/dead probe (extrinsic fluorescence) as well as the fluorescence intensity of NADH (intrinsic fluorescence). EBs were assessed at

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Supporting Information available online

Author contribution

All authors met the criteria defined by the Vancouver Guidelines on authorship. Namely, D.G.B. contributed to the design of the study, was primarily responsible for data acquisition and analysis and participated in the drafting and revising of the manuscript. J.M.S. contributed to the conception and design of the study and was instrumental in drafting and revising the manuscript. J.J.F. made substantial contributions to data acquisition and analysis and participated in the revising of the manuscript. K.W.E. contributed to the design of the study, provided insight related to the interpretation of the data and participated in the drafting and revising of the manuscript. B.M.O. contributed to the design and implementation of the study, provided insights related to the interpretation of the data and participated in drafting and revising the manuscript. All authors approved the final version of the manuscript to be submitted.

Conflict of interest statement

The authors have declared no conflict of interest.

subsequent time points (days 6–12) for the formation of beating areas as an indicator of functional differentiation.

Results—Statistical comparison indicated a strong positive correlation between extrinsic fluorescence intensity of the live/dead stain and intrinsic fluorescence of NADH, suggesting that the intensity of NADH fluorescence could be used to reliably and non-invasively assess death of cells of EBs. Furthermore, EBs that had high levels of cell death soon after aggregate formation had limited ability to give rise to functional cardiomyocytes at later time points.

Conclusions—We demonstrate the utility of NADH fluorescence intensity as a non-invasive indicator of cell death in stem cell aggregates when measured using multi-photon excitation. In addition, we show that the degree of stem cell death at early stages of differentiation is predictive for the formation of functional cardiomyocytes.

Keywords

Autofluorescence; Cell aggregate; NADH; Stem cells; Viability

Introduction

Cell death is essential for the development, homeostasis and repair of tissues (reviewed in Doseff, 2004; Meier et al., 2000). Recent studies suggest cell death is equally important for pluripotent stem cells and ‘quasi-developmental’ events that occur with differentiation (Edel et al., 2010; Kawamura et al., 2009; Li et al., 2011; Marion et al., 2009; Nicolaije et al., 2012; Zhao et al., 2008). For example, inhibition of prostate apoptosis response-4 in pluripotent stem cells limits differentiation of cells of endoderm, mesoderm and ectoderm lineages (Bieberich et al., 2004). However, greatly limiting the study of the significance of stem cell death has been the inability to distinguish dead cells from viable cells without application of exogenous labels that might alter stem cell state. Thus, methods for non-invasive detection of cell death are necessary. Furthermore, stem cells are maintained and differentiate most efficiently in stem cell aggregates and so it is crucial to assess cell death without disrupting the aggregate (Ohgushi et al., 2010).

The intensities of autofluorescent metabolites have been used as biomarkers of cell viability in yeast (Liang et al., 2007), primary epithelial cells (Levitt et al., 2006) and kidney tissue (Luger-Hamer et al., 2009). Moreover, fluorescence lifetime of intrinsic fluorophores has been utilised as an indicator of cell death (Guo et al., 2011; Yu et al., 2011) and as a means for distinguishing between modes of cell death (Su et al., 2011; Wang et al., 2008; reviewed in Wang et al., 2009a). Of particular interest is the metabolite nicotinamide adenine dinucleotide (NADH), a critical co-enzyme in cell energy metabolism. NADH is a primary electron transporter, especially in oxidative phosphorylation, through which significant ATP is produced (Ying, 2008). Given this role, the concentration of NADH in the cell cytoplasm (including mitochondria) would likely decrease with oxidative injury. Flow cytometry, spectroscopy and fluorescence imaging-based studies indicate that NADH levels do decrease with reversible hypoxic injury (*i.e.* when oxygen is restored, the cells recover native function) (Levitt et al., 2006; Liang et al., 2007; Luger-Hamer et al., 2009). However, with irreversible injury, there is actually a sharp, transient increase, followed by a variable

decrease in NADH levels (Liang et al., 2007). Compelling fluorescence image data suggest that increases in NADH correspond to a sub-population of mitochondria which occupy the perinuclear space (Levitt et al., 2006). These mitochondria are thought to mobilise in response to apoptotic stimuli and may provide the energy or other, as yet undefined functions needed to complete apoptosis. This direct correlation between cell death and changes in NADH levels, which can be assessed through changes in endogenous fluorescence of living cells, suggest that this endogenous fluorescence could be utilised as a non-invasive tool for monitoring viability of individual cells and aggregates, including the viability of stem cells.

Therefore, the goal of this study was first to determine whether changes in intensity of NADH fluorescence correlate with cell death in stem cell aggregates and second to determine the physiological impact of early stage death of stem cells on long-term function of stem cell progeny. We utilised multi-photon laser scanning microscopy (MPLSM) to detect endogenous NADH fluorescence in stem cell aggregates because of its improved deep sectioning, viability maintenance and signal-to-noise capabilities compared with traditional optical approaches. Detection of NADH fluorescence with conventional, single-photon fluorescence microscopy (*i.e.* epifluorescence or confocal microscopy) is limited by depth of light penetration into the sample and lack of tunability in the laser sources to maximise detection of intrinsic fluorescence. In addition, excitation of cell aggregates in the ultraviolet range with single photons can be cytotoxic (Lisby et al., 2005). MPLSM is a non-linear optical sectioning technique that requires absorption of two or more low-energy photons in the near-infrared range for effective fluorophore excitation (Denk et al., 1990). Sufficient energy for two-photon excitation is only present at the plane of focus such that, unlike other fluorescence microscopy approaches, no out-of-plane signal interference or photobleaching occurs. Along with the fact that these longer wavelengths of light are more immune to scattering and are less phototoxic (Squirrell et al., 1999), the effective imaging depth can greatly exceed conventional confocal microscopy (Centonze and White, 1998). Effective imaging depth is especially important for analysing embryoid bodies (EBs), the common spherical, multi-cellular intermediate between pluripotent stem cells (*e.g.* embryonic stem cells [ESCs] or induced pluripotent stem cells [iPSCs] and mature cells. The typical EB size range is approximately 100–500 μm in diameter and so fluorescence signals that may be generated within the EB are difficult to detect with other optical imaging methods. In addition, we are interested in determining the ability to analyse NADH fluorescence in an enhanced-throughput fashion using our recently developed multi-photon flow cytometry (MPFC) system (Buschke et al., 2011), which is compatible with the NADH approaches described in the ‘Materials and methods’ section. Thus, we generated mouse EBs and probed a single-optical section in the interior of the intact EB soon after formation for NADH expression using MPLSM, and then monitored the same EBs for several days noting the presence or absence of beating cardiomyocytes.

Results

Induction of cell death in EBs via ATP-competitive kinase inhibition

To assess the relationship between cell death and increased live/dead probe intensity, we controllably increased the degree of cell death of murine ESC-derived EBs by treating with graded concentrations of staurosporine, ranging from 0 to 500 nM. Staurosporine is an alkaloid compound produced by *Strep-tomyces staurosporeus* which acts as an ATP-competitive kinase inhibitor, thus promoting caspase 3 expression and inducing apoptosis in a variety of cell types (Karaman et al., 2008). It should be noted that although staurosporine is a potent inducer of apoptosis, it is very likely that other types of cell death, such as necrosis and autophagy, also occur within the cell aggregates with and without staurosporine treatment. Here, EBs were treated with staurosporine 3 days after EB formation. Following treatment, EBs were imaged at a single time point to detect extrinsic fluorescence of the live/dead probe from a single-optical section using MPLSM (Figure 1A). A strong correlation existed between the percentage of dead and dying cells of the EB and the mean intensity per pixel ($r = 0.87$; $P < 0.0001$), that is, the intensity of the probe (mean intensity per pixel) increased as the percentage of dead cells increased (Figure 1B and 1C). Therefore, subsequent comparisons between groups were based on mean intensity per pixel for each EB as a measure of cell death. Next, to determine whether cell death within an EB could be predictably altered with staurosporine treatment, the dose-response was determined by measuring the mean intensity per pixel of the live/dead probe in EBs either without treatment or with 100, 200 and 500 nM staurosporine exposure. Mean intensity per pixel increased with increasing staurosporine exposure, indicating that staurosporine can be used to reproducibly induce cell death in EBs (Figure 1D).

NADH fluorescence intensity increases with increased cell death in EBs

Using staurosporine treatment to induce cell death, we tested whether levels of intracellular NADH endogenous intensity were indicative of cell death in EBs, using a well-established, extrinsic, live/dead probe as the gold standard to assess cell death (Figure 2A). Others have shown that NADH autofluorescence increases dramatically in the first few hours after induced cell death and subsequently drops below baseline levels (Liang et al., 2007). We found the same to be true for cells of EBs and, specifically, that NADH intensity peaked approximately 3 h following staurosporine treatment (Supplementary Figure S1). Thus, in order to monitor changes at or near the peak levels of NADH intensity, EBs were imaged at 3 h following staurosporine (0, 100 and 500 nM) treatment to induce cell death. Just prior to imaging, the extrinsic live/dead probe was applied and then EBs were placed into individual wells of gelatin-coated, multi-well microscopy slides. Each EB was imaged on the MPLSM at 890 nm with a 520/35 bandpass filter for the live/dead probe and next imaged at 780 nm with a 457/50 bandpass filter for NADH expression at the same optical plane. Independent spectrofluorometric analysis indicated that NADH is the principal contributor to fluorescence emission under these conditions (Bird et al., 2005; Lakowicz, 2006) (Supplementary Figure S2). Although flavin adenine dinucleotide and lipoamide dehydrogenase could be excited at 780 nm (Huang et al., 2002) and contribute to the intrinsic intensity of the EBs, the 457/50 emission filter used in these studies helps ensure the contribution is overwhelmingly from NADH and again matches known spectral profiles

for NADH. In addition, extensive testing of control cultures revealed no overlap of fluorescence intensity between fluorophores (Supplementary Figure S3). Mean intensity per pixel was determined for both NADH (intrinsic intensity) and live/dead probe (extrinsic intensity) and plotted relative to each other (Figure 2B). To determine statistical dependence of NADH expression on the presence of dead cells (as defined by the live/dead extrinsic probe), a Pearson's correlation coefficient was calculated and found to be statistically significant ($r = 0.69$; $P < 0.0001$). This trend was maintained when individual treatment groups were considered separately. Thus, NADH intensity reflected cell death similar to the live/dead probe and, therefore, could be used as a non-invasive biomarker of cell death in EBs.

Low levels of NADH fluorescence intensity of EBs soon after formation are predictive of functional differentiation of cardiomyocytes

Cell death is crucial for development and homeostasis *in vivo*, including in the heart (Poelmann et al., 2000; Sheng et al., 1997; Yeh et al., 1998). The physiological relevance of cell death with the 'quasi' development that occurs with stem cell differentiation *in vitro* has not been investigated. This is an especially challenging task as, unlike development, not all EBs give rise to the same cell types and the arrangement varies substantially. Thus, a non-invasive biomarker of cell death in intact EBs, as we have shown above, is particularly useful for discerning the impact of cell death (especially at early phases) on the subsequent development of particular cell types and tissues. Here, we investigate the impact of early cell death on cardiomyocyte differentiation and function. To this end, EBs were treated with staurosporine 3 days after EB formation. Following treatment, EBs were imaged at a single time point to detect extrinsic fluorescence of the live/dead probe and intrinsic fluorescence of NADH from a single-optical section of individual EBs using MPLSM (Figure 3A). Each EB was then monitored daily for 9 days (12 days after EB formation) for the presence of beating areas indicative of cardiomyocyte differentiation. At day 12 after EB formation, EBs were probed for cardiac troponin T (cTnT) expression via immunofluorescence, to confirm the cardiomyocyte phenotype (Figure 3B and 3C). All beating areas detected exhibited corresponding positive staining for cTnT. EBs that formed beating areas comprised 44% of the total population. The mean intrinsic and extrinsic fluorescence intensity of each EB were plotted and those EBs that formed beating areas were distinguished from those that did not (Figure 3D). The EBs that gave rise to beating areas appeared to have low levels of both intrinsic and extrinsic fluorescence intensity. Indeed, both the intrinsic and extrinsic fluorescence intensity levels of EBs that formed beating areas versus those that did not form beating areas were statistically different ($P < 0.01$). Interestingly, if an arbitrary intensity level of 1.0 (normalised arbitrary intensity units) was selected, 88% of EBs that formed beating areas had a mean NADH intensity per pixel, at day 3, below that level and similarly, 84% of EBs that formed beating areas had a mean live/dead probe intensity per pixel, at day 3, below that level. Thus, the population of EBs that formed beating areas could be enriched twofold by selecting EBs with low endogenous fluorescence intensity. Of note, EBs from all three treatment groups (0, 100 and 500 nM) contributed to the total number of beating areas (75%, 22% and 3% of beating EBs, respectively; $n > 20$ EBs for each concentration). Furthermore, within the group of EBs that developed beating areas, those with lower levels of either intrinsic or extrinsic fluorescence (*i.e.* less cell death) were likely to form beating

areas sooner. EBs imaged on day 3 that formed beating areas by day 6 exhibited statistically lower levels of NADH and live/dead fluorescence intensity than EBs that formed EBs on day 12 or did not form beating areas. Similarly, EBs imaged on day 3 that formed beating areas by day 9 exhibited statistically different levels of NADH and live/dead fluorescence intensity than EBs that did not form beating areas (Figure 3E). This trend was maintained even without treatment with staurosporine when comparing EBs that formed beating areas by day 6 (mean intrinsic intensity/pixel 0.78 ± 0.11) to those that did not form beating areas by day 6 (mean intrinsic intensity/pixel, 0.81 ± 0.07). Thus, NADH levels determined at early time points may be predictive of the temporal onset of functionally differentiated cells of EBs and so might be used as a biomarker for non-invasive analysis and purification of stem cell aggregates for both research and clinical application.

Discussion

In this study, we show that the intensity of NADH fluorescence changes in response to staurosporine-induced cell death in murine EBs and so might be used to reliably and non-invasively assess viability of EBs. Increased NADH expression after induction of cell death has been observed in other cell types (Levitt et al., 2006; Liang et al., 2007; Luger-Hamer et al., 2009), although this is the first study to show such a response in intact cell aggregates. Furthermore, we show that the mean intensity per pixel of NADH fluorescence is a predictive indicator of the propensity for functional cardiomyocyte differentiation in EBs.

We have focused here on analysis of EBs, as there is still much to learn from these aggregates in terms of the spatial and temporal cues that give rise to particular differentiated cell types (Durrans and Stuhlmann, 2010; Mochizuki et al., 2011; Shukla et al., 2010; Takei et al., 2009). We were particularly interested in the impact of severe cellular stress on later, long-term function of mature cells types. Future studies could more effectively tap the ability to track intrinsic fluorescence of the same EB over time, by assessing the progressive contribution of cell death to the developing EB. It is possible that cell death at localised and low levels may be beneficial and not detrimental in developing EB. Similar approaches could be applied to any cell aggregate, including tumour spheroids, neurospheres, cardiospheres, lymphoblastoid cells and perhaps most interestingly to *in vitro*, three-dimensional engineered tissue models. One primary goal of engineered tissue models is to present cues in a more defined manner than that of the heterogeneous EB (reviewed in Lund et al., 2009; Lutolf et al., 2009), and non-invasive assessment of cell death could advance these efforts tremendously. We emphasise here the utility of NADH as a biomarker of stem cell death in cell aggregates; however, the approach is certainly applicable to analysis of single-stem cells as well. In this case, single-photon fluorescence microscopy and traditional flow cytometry techniques might be effectively employed because sample thickness is relatively small. In all cases, the spectral profiles in both excitation and emission should be carefully considered and experimental method accordingly adjusted so as to avoid signal from alternative sources of intrinsic fluorescence that could include cellular elements, extracellular elements or even certain small molecules used to induce cell death (*e.g.* camptothecin) that fluoresce in the same region of the emission spectrum as NADH.

Changes in NADH intensity with cell death are time dependent and so would most effectively align with changes in cell death soon after induction of trauma or exposure to signalling molecules that promote cell death. Time dependence of NADH expression is beneficial in conditions of induced cell stress, as analyses can be orchestrated to correspond with peak expression. However, in the course of typical culture, stem cell aggregates exhibit constant, low level, cell death that should be detectable at any given time point. In other words, the large number of cells analysed (as opposed to a single cell) ensures a high probability of accurately capturing the global health of the EB. Indeed, EBs without staurosporine treatment that ultimately give rise to beating areas exhibit the same trend of lower mean intrinsic fluorescence intensity than EBs that do not generate beating areas.

It should be noted that the distribution pattern of fluorescence intensity of the intrinsic fluorophores and extrinsic markers are not alike. The extrinsic dye yields a punctate pattern whilst the intrinsic fluorescence is diffuse. This difference likely reflects the difference not only in binding target but also in the temporal window of cell death associated with each 'probe'. The extrinsic marker identifies cells with permeable membranes, likely at the later stages of cell death. In contrast, the autofluorescent intensity corresponds to fluctuations of NADH that can occur at both early and late stages of cell death and does not necessarily imply a compromised cell membrane. In addition, the heterogeneity of cell types comprising the aggregates may be more or less resistant to cell death and associated membrane permeability (extrinsic probe), whereas even those cell types more resistant to cell death will exhibit fluctuations in NADH in response to stressful stimuli (*i.e.* staurosporine treatment).

Another important consideration is that NADH amount and state in healthy stem cells and their progeny can be altered as a consequence of other cell functions (Heikal, 2010), especially differentiation (Guo et al., 2008; Hronik-Tupaj et al., 2011; Konig et al., 2011; Reyes et al., 2006; Rice et al., 2007; Rice et al., 2010; Uchugonova and Konig, 2008). A variety of studies suggest that pluripotent stem cells are metabolically distinct from differentiated cells (Cho et al., 2006; Ezashi et al., 2005; Kondoh et al., 2007; Wang et al., 2009b). As one example, pluripotent stem cells can proliferate, and may preferentially maintain pluripotency, in a hypoxic environment (Francis and Wei, 2010). These studies suggest that stem cells are preferentially using, or at least are capable of using, non-oxidative pathways as opposed to oxidative phosphorylation predominantly employed by most mature cell types. Thus, we and others have begun to investigate the concept that intrinsic fluorescence signatures, including fluorescence intensity and fluorescence lifetime (Lakowicz et al., 1992a) of bound and free NADH (Lakowicz et al., 1992b) in particular, could be used to identify changes with stem cell differentiation. Specifically, human mesenchymal stem cells show an increase in NADH fluorescence intensity and the fluorescence lifetime of bound NADH as they differentiate towards bone and fat lineages (Rice et al., 2010). In addition, discernable differences in fluorescence intensity and lifetime can be identified with differentiation of salivary gland stem cells (Konig et al., 2011). Thus, it is clear that intrinsic fluorescence of metabolites can serve to distinguish viable cells from dead cells and mature cells from progenitors. Lacking are comprehensive studies and robustly defined parameters to distinguish each stem cell state (*e.g.* viable, proliferative, migratory and differentiating) and sub-states (*e.g.* cell death due to apoptosis, necrosis and autophagy). However, the work presented here takes one clear step towards defining

parameters for viability of stem cells at time points when the majority of cells of EBs are still in a pluripotent or at least multi-potent state.

Here, we identify consistent increases in NADH fluorescence intensity with induced cell death in EBs and find that the percentage of EBs that give rise to functional cardiomyocytes is higher in EBs that had lower fluorescence intensity, either endogenous or exogenous, soon after EB formation. On the basis of these results, we envision defining and then applying the derived indices to a novel fluorescence excitation MPFC system recently developed in our laboratory (Buschke et al., 2011). This system can accurately probe cells in the interior of intact multi-cell aggregates or tissue constructs in an enhanced-throughput manner and measure corresponding fluorescence properties, including intrinsic fluorescence properties. In addition, this system is being adapted to purify populations based on user-defined fluorescence indices, making it feasible to enrich for stem cell aggregates with specific intensity profiles. Enriched populations can be used for molecular profiling to rapidly and comprehensively analyse transcriptional changes with alterations in cell death and how these changes impact on cell behaviour including propagation and differentiation. This technology, and others like it (reviewed in Buschke et al., 2010), coupled with the knowledge gained in this study, has tremendous potential for both basic research and clinical cellular transplantation in that the structure of the aggregate can be maintained, exogenous labels avoided and purification achieved in an enhanced-throughput fashion.

Materials and methods

Stem cell culture

HM1 mouse ESCs (Open Biosystems) were cultured in Dul-becco's modified Eagle's medium (DMEM + Glutamax; Invitrogen) with 10% foetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (Lonza), 1% non-essential amino acids (Invitrogen) and 0.0007% (1% of a 35 ml/50 ml solution) β -mercaptoethanol (MP Biomedicals LLC). To maintain pluripo-tency, media were supplemented with leukemia inhibitory factor (LIF; Millipore) at 2000 U/ml and bone morphogenic protein 4 (BMP-4; R&D Systems) at 10 ng/ml (Ying et al., 2003). EBs were made via the hanging drop method (day 0) (Maltsev et al., 1993). ESCs were harvested and resuspended in DMEM + 10% FBS (no LIF or BMP-4) at 1.6×10^4 cells/ml. This cell suspension was used to make 30 μ l hanging drops over $1 \times$ PBS in 100 mm Petri dishes. EBs were harvested 3 days after formation (day 3); this stage served as the time point for initiation of all studies described here.

Drug treatment and exogenous viability staining

For induction of cell death, staurosporine (Sigma–Aldrich) was applied to EB populations at concentrations ranging from 100 to 500 nM for 2 h. EBs were stained with LIVE/DEAD[®] Fixable Dead Cell Stain Kit (L23101, excitation, 495; emission, 520; Invitrogen) at a 1:1000 dilution in $1 \times$ PBS for 10 min at room temperature on a rocking platform. EBs were rinsed once with $1 \times$ PBS and resuspended in DMEM + 10% FBS. This probe penetrates dead or dying cells and binds to intracellular amines throughout the cell volume. The probe also binds extracellular amines of live cells, but is restricted to the cell border and so generates a signal approximately 100-fold lower than that of dead cells (manufacturer's specifications;

Invitrogen). After staining, individual EBs were plated on 0.1% gelatin-coated 9×2 microslides (ibidi) for imaging and for subsequent tracking for beating cardiomyocytes.

MPLSM imaging

Embryoid bodies were imaged using MPLSM with optical configuration similar to that previously reported (Buschke et al., 2011). To view the live/dead probe, the Ti-Sapphire laser was tuned to 890 nm with a 520/35 nm emission filter. To view NADH intensity, the Ti-Sapphire laser was tuned to 780 nm excitation with a 457/50 nm bandpass emission filter. Average power measurements were taken at the sample, and determined to be 21 and 37 mW, for 890 and 780 nm excitation conditions, respectively, which is within a reported range supportive of cell viability (Dela Cruz et al., 2010). Images were taken with a Plan Apo VC 20 $\times$ air, 0.75 NA, 1.0 mm WD objective (Nikon) at a resolution of 512×512 with a one second exposure time per scan, corresponding to a dwell time of 3.8 μ s/pixel. Power and gain settings were set such that less than 5% of pixels were saturated and background noise was kept to a minimum. Brightfield and intensity images were collected using in-house developed software (WiscScan). Fluorescence and size measurements of EBs were made using ImageJ software (Collins, 2007). For analysis, background levels were identified such that less than 10% of pixels on a background image (*i.e.* containing no EBs) were saturated for all conditions. Background fluorescence intensity levels were subtracted from each pixel of the EB image (background corrected [BC]). Next, a region of interest (ROI) was created to match the border of EB cross-section of the BC intensity image. The percentage of viable cells of an EB was determined by first identifying the fluorescence intensity level of a living cell. The fluorescence intensity level of living cells was then subtracted (live cell corrected [LCC]) from each pixel of the ROI defined above. The percentage of viable cells of the EB was defined as the fraction of pixels without intensity after LCC was divided by the total number of pixels per ROI. The percentage of dead cells of the EB was defined as the fraction of pixels with intensity after LCC was divided by the total number of pixels per ROI. The mean intensity per pixel was defined as the total intensity of the ROI divided by the total number of pixels in the ROI.

Assessment for cardiomyocyte function and immunofluorescence staining

Imaged EBs were placed in an incubator overnight and analysed for attachment on the following day (day 4). Each subsequent day, medium was replenished in the microwell slides and each EB was scored for formation of beating areas. On day 12 of differentiation, EBs were fixed with 4% paraformaldehyde and stained for cTnT. The National Stem Cell Bank protocol (www.wicell.org, SOP-CH-210C) for immunolabelling of cardiac markers in EBs was utilised for this assessment. Mouse anti-TnT (cardiac isoform Ab-1, cTnT, clone 13-11; Fisher Scientific) primary antibody and goat anti-mouse Alexa Fluor 568 (Molecular Probes/Invitrogen) secondary antibody were used. Fluorescence images were acquired using a Zeiss Axiovert 40CFL inverted microscope with a 4 $\times$ objective, excitation filter 560/55 nm and emission filter 645/75 nm.

Spectrofluorometry

A Fluorolog-3 Spectrofluorometer (Horiba Scientific) was utilised to determine the fluorescence emission spectrum of un-manipulated mouse embryonic stem cells. The

excitation wavelength was set at 350 nm, corresponding to the excitation peak of NADH, whereas intensity measurements were recorded within a range of 400–600 nm, corresponding to the emission spectrum of NADH. HM1 mouse embryonic stem cells were harvested and resuspended in $1 \times$ PBS before measurement. β -NADH (Sigma–Aldrich) was reconstituted in $1 \times$ PBS at a concentration of 7 mM.

Statistical analysis

For comparison of intrinsic (NADH) and extrinsic (live/dead probe) fluorescence intensity levels of cell aggregates at early and late time points and between groups treated with staurosporine, one-way analyses of variance and Student's *t*-test were used. Data were reported as average $\pm$ standard deviation and were analysed with JMP 5.0.1 for Windows (SAS Institute, Inc.). Pearson correlation coefficient (*r*) and confidence interval (*P*) were used to determine whether linear correlations existed between paired values corresponding to (i) the percentage of viable cells in an EB and mean extrinsic fluorescence intensity (live/dead probe) per pixel and (ii) mean intrinsic (NADH) fluorescence intensity and mean extrinsic (live/dead probe) fluorescence intensity. A 95% confidence (*P* < 0.05) interval was applied for statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BC	background corrected
cTnT	cardiac troponin T
EBs	embryoid bodies
ESCs	embryonic stem cells
LCC	live cell corrected
MPFC	multi-photon flow cytometry
MPLSM	multi-photon laser scanning microscopy
MSCs	mesenchymal stem cells
NADH	nicotinamide adenine dinucleotide

ROI region of interest

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Figure 1. Induction of cell death via staurosporine

(A) Schematic depicting the experimental approach used to determine whether cell death could be reliably induced with staurosporine. EBs were generated from HM1 embryonic stem cells and treated with staurosporine 3 days after EB formation. Following treatment, EBs were imaged at a single time point to detect extrinsic fluorescence of the live/dead probe from a single-optical section using MPLSM. (B) A strong positive correlation existed between the percentage of dead cells of the EB and the mean intensity per pixel ($r = 0.87$; $P < 0.0001$). Each dot represents a single EB. The percentage of dead cells of the EB and the

mean intensity per pixel were determined as described in the Materials and methods section. Data shown correspond to one experiment and are representative of the three experiments conducted. The range in EB intensities within treatment groups for this experiment was 0.8–1.2, 0 nM; 0.8–1.1, 100 nM; 0.8–1.1, 200 nM; 1–1.4, 500 nM. **(C)** Representative images of live/dead staining of EBs with and without staurosporine treatments. Background correction (BC) indicates that background fluorescence levels have been subtracted from the image. White lines indicate the outline of an EB. Live cell correction (LLC) indicates that fluorescence associated with living cells has been subtracted from the image. Scale bar = 200 μ m. **(D)** Mean intensity of the live/dead probe increased with increasing concentrations of staurosporine. * $P < 0.05$ when compared with the intensity of EBs without staurosporine treatment.

Figure 2. Relationship between extrinsic fluorescence intensity of a live/dead probe and intrinsic fluorescence intensity of NADH

(A) Schematic depicting the experimental approach designed to determine whether NADH intensity correlates with cell death. EBs were generated and treated with staurosporine 3 days after EB formation. Just prior to imaging, the extrinsic live/dead probe was applied and then EBs were placed into individual wells of gelatin-coated, multi-well microscopy slides. Each EB was imaged on the MPLSM at 890 nm with a 520/35 bandpass filter for the live/dead probe and next imaged at 780 nm with a 457/50 bandpass filter for NADH expression at the same optical plane. (B) Correlation between mean intrinsic fluorescence intensity per pixel of EBs and mean extrinsic fluorescence intensity per pixel. A strong correlation ($r = 0.69$; $P < 0.0001$) exists between the known indicator of cell death (live/dead probe) and NADH fluorescence intensity. Each dot represents a single EB imaged at the same plane for both intrinsic and extrinsic fluorescence using MPLSM. (C) Representative images of

NADH intensity of EBs and live/dead staining with (500 nM) and without (0 nM) staurosporine treatment. Brightfield image is wide field and fluorescent images are optical sections. Scale bar = 200 μm .

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Figure 3. Intrinsic fluorescence intensity of NADH as a predictive index of cardiomyocyte differentiation

(A) Schematic depicting the experimental approach designed to determine whether NADH intensity levels at an early time point after EB formation are indicative of later function of differentiated cardiomyocytes. EBs were treated with staurosporine 3 days after EB formation. Following treatment, EBs were imaged at a single time point to detect extrinsic fluorescence of the live/dead probe and intrinsic fluorescence of NADH from a single-optical section of individual EBs using MPLSM. Each EB was then monitored daily for 9

days (up to 12 days after EB formation) for the presence of beating areas indicative of cardiomyocyte differentiation. **(B)** Microscopic observation of a beating area. Shown is a Brightfield image with the beating region outlined in black. **(C)** Cardiac troponin T (cTnT) staining corresponds to microscopic observation of beating behaviour **(B)**. EBs were formed and plated in 9 x 2 microslides. Upon observation of beating areas, immunofluorescence staining for cTnT was performed. In all cases where beating cells were observed, cTnT staining was also observed. Shown is the outline of the beating area and corresponding cTnT staining (red) superimposed on the Brightfield image. Scale bar = 200 μ m. **(D)** Distribution of EBs which give rise to beating areas at 6–12 days after EB formation and corresponding levels of intrinsic fluorescence intensity of NADH and extrinsic fluorescence of the live/dead probe detected 3 days after EB formation. **(E)** Comparison of mean intrinsic fluorescence intensity of NADH of EBs and the time after EB formation that beating areas appear. EBs with low mean intensity per pixel of intrinsic fluorescence intensity of NADH soon after EB formation are more likely to give rise to beating cardiomyocytes at later time points than those with high levels early on (* $P < 0.05$).