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A highly selective fluorescent probe for direct detection and isolation of mouse embryonic stem cells

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ABSTRACT

Stem cell research has gathered immense attention in the past decade due to the remarkable ability of stem cells for self-renewal and tissue-specific differentiation. Despite having numerous advancements in stem cell isolation and manipulation techniques, there is a need for highly reliable probes for the specific detection of live stem cells. Herein we developed a new fluorescence probe (**CDy9**) with high selectivity for mouse embryonic stem cells. **CDy9** allows the detection and isolation of intact stem cells with marginal impact on their function and capabilities.

Stem cells are unique cell populations found in almost every multi-cellular organism with the capacity to give rise to many different types of cells in the body during early development and growth.[1–3] Embryonic stem cells (ESC) are isolated from very young embryos (3.5 days-old) and found in the inner cell mass of blastocysts (i.e., embryos consisting of around 100 cells) of fertilized eggs.[3] ESC are pluripotent as they are able to remain undifferentiated in vitro and then differentiate into all the different cell types of the three germ layers when given the correct cues.[4]

Despite the potential of stem cells for the treatment of complex diseases, there are still no effective ways to detect stem cells in vivo or ex vivo. This is mainly due to their heterogenetic nature and unpredictable pattern of proliferation and differentiation in ex vivo cultures.[2] Current characterization methods for stem cells mainly rely on immunohistochemistry using protein markers and subsequent treatment with secondary antibodies.[5] These methods typically require fixation of the cells with paraformaldehyde, which hampers the application of the cells in subsequent studies. Therefore, there is a need for new strategies that allow direct detection and monitoring of stem cells in a non-invasive manner.

Fluorescence imaging offers many advantages for non-invasive cell tracking. On top of being rapid and extremely sensitive, fluorescent probes are compatible with a broad range of instrumentation.[6] In the last decade, considerable effort has been put into the development of highly sensitive fluorescent molecular imaging tools[7,8] and genetically encoded fluorescent protein reporters.[9] These reporters have proven their exceptional value in numerous biological studies, but entail some disadvantages, such as the potential interference with protein function and the need for genetic manipulation. An alternative to encoded protein reporters for labeling cells in vivo are small molecule fluorescent probes.[10,11] Our group has pioneered the development of fluorescent probes using the Diversity Oriented Fluorescence Library Approach (DOFLA). DOFLA exploits the power of combinatorial chemistry to derivatize fluorescent scaffolds with several functional groups and generate libraries of structurally and spectrally diverse fluorescent molecules.[12] To date, DOFLA has been an excellent source for the discovery of unique sensors and probes, especially for targets with limited molecular information.[13–15] In the context of stem cell probe development, three fluorescent probes for mouse embryonic stem cells (mESC) have been previously identified by the DOFLA: compound of designation yellow 1 (**CDy1**), compound of designation green 4 (**CDg4**) and compound designation of blue 8 (**CDb8**).[16–18] **CDy1** was the first mESC-staining probe to be reported from DOFLA, and it is a rosamine-based fluorophore that labels

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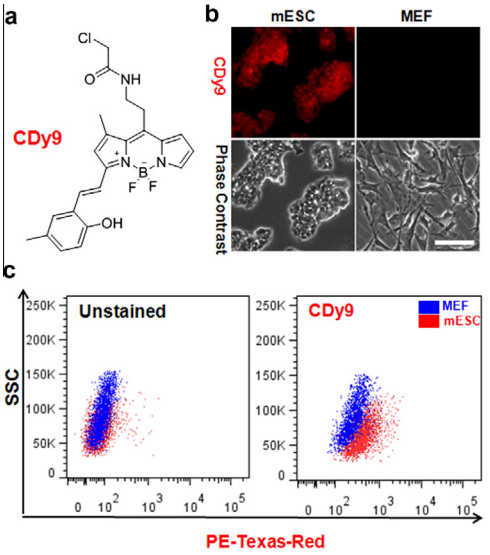
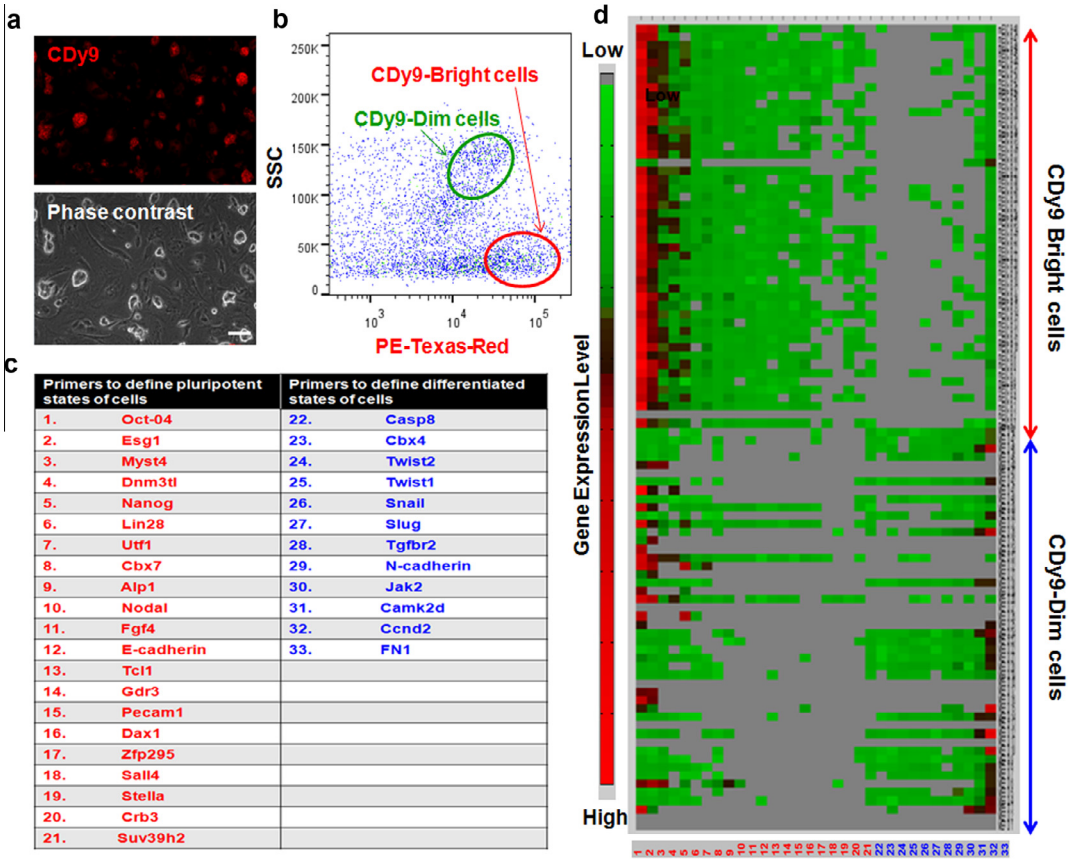


Figure 1. CDy9 is a novel and versatile mESC-specific fluorescent probe. (a) Chemical structure of **CDy9**. (b) **CDy9** selectively stains mESC over MEF. Both mESC and MEF were incubated with 1 μM **CDy9** and imaged under the fluorescence microscope after 1 h. Scale bar: 25 μm. (c) Flow cytometry analysis of mESC and MEF after incubation with **CDy9**. The fluorescence intensity of mESC upon treatment with **CDy9** is brighter than in MEF (right dot plot) and in unstained mESC (left dot plot).

mESC as well as mouse induced pluripotent stem cells (iPSC). **CDy1** can stain mouse iPSC in early stages of development, allowing early detection and characterization of these cells.[19,20] **CDb8** was reported as a mESC-staining probe with short emission wavelengths ($\lambda_{exc.}/\lambda_{em.}$:369 nm/487 nm) discovered upon the combinatorial derivatization of a xanthone fluorescent scaffold. Finally, **CDg4** is a chalcone derivative that labels mESC upon binding to the glycogen molecules present on the surface of mESC. Notably, despite the identification of these three fluorescent probes with preferential labeling of mESC over mouse embryonic fibroblasts (MEF), we observed that **CDy1**, **CDb8** and **CDg4** can also stain differentiated cells from various lineages (i.e., ectoderm, endoderm and mesoderm) and therefore cannot be used for direct and unequivocal identification and isolation of mESC.

Herein we report the identification of a fluorescent small molecule with high specificity for mESC and marginal staining in a whole range of cells derived from different lineages of the three germ layers. From our high-throughput cell imaging screening, we identified **CDy9** (compound of designation yellow 9, $\lambda_{exc.}/\lambda_{em.}$: 563 nm/578 nm) as a fluorescent small molecule with high selectivity for mESC over MEF (Fig. 1). **CDy9** was synthesized by derivatization of the 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (BODIPY) fluorescent scaffold using solid-phase synthesis (see Supporting information for synthetic and characterization data).[21] In order to identify the chemical groups of **CDy9** that were responsible for its preferential labeling of mESC, we evaluated the fluorescence staining of mESC and MEF upon incubation with two



Primers to define pluripotent states of cells		Primers to define differentiated states of cells	
1.	Oct-04	22.	Casp8
2.	Esg1	23.	Cbx4
3.	Myst4	24.	Twist2
4.	Dnm3tl	25.	Twist1
5.	Nanog	26.	Snail
6.	Lin28	27.	Slug
7.	Utf1	28.	Tgfbr2
8.	Cbx7	29.	N-cadherin
9.	Alp1	30.	Jak2
10.	Nodal	31.	Camk2d
11.	Fgf4	32.	Ccnd2
12.	E-cadherin	33.	FN1
13.	Tcl1		
14.	Gdr3		
15.	Pecam1		
16.	Dax1		
17.	Zfp295		
18.	Sall4		
19.	Stella		
20.	Crb3		
21.	Suv39h2		

Figure 2. Isolation of CDy9-stained cells and analysis by single cell PCR. (a) **CDy9** selectively stains mESC in co-cultures with MEF. Co-cultures were incubated with 1 μM **CDy9** and imaged under the fluorescence microscope after 1 h. Scale bar: 100 μm. (b) FACS analysis of co-cultured mESC and MEF after **CDy9** staining. *X*-Axis refers to the **CDy9** fluorescence intensity and *Y*-axis refers to the side scattering (SSC), an indication of the size of the cells. FACS enabled the isolation of **CDy9**-bright and **CDy9**-dim populations. (c) Table of primers used to distinguish pluripotent (red) from differentiated (blue) cells. (d) Heatmap obtained from single cell PCR analysis of **CDy9**-bright and **CDy9**-dim populations. **CDy9**-bright cells display an up-regulation of pluripotent genes (mESC-like) and **CDy9**-dim cells show an up-regulation of differentiated genes (MEF-like).

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