

A Fluorescent Rosamine Compound Selectively Stains Pluripotent Stem Cells**

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Stem cells, which are capable of self-renewing and differentiating into various types of cells, have captured great interest as a valuable resource for regenerative medicine and developmental biology research. Technical progress during the last decade has enabled the isolation of stem cells from a wide range of tissues, their differentiation into specific types of cells, and the generation of induced pluripotent stem cells (iPSC) from somatic cells. The recent success of patient-

specific iPSC generation^[1] and their differentiation into functional cells^[2] exemplifies how stem cells can be used for drug discovery and treatment of specific patients having complex diseases.^[2]

However, despite the general enthusiasm for the multiple applications of stem cells, their practical use both in research and disease therapy has been hampered by the heterogeneity of stem cells and their unpredictable proliferation and differentiation.^[3] The current methods of isolation and characterization of stem cells mostly depend on their morphology in the culture, such as colony or sphere formation, and immunodetection of marker proteins. These methods, however, require extended times and antibody reactions which may make the cells unsuitable for further usage. Therefore, the development of tools and technologies that may facilitate the isolation, identification, and characterization of stem cells is one of the most demanding requisites in the field of stem-cell research and applications.

Fluorescent small molecules have been widely used for the visualization of polymeric biomolecules or cellular organelles.^[4] We have employed combinatorial chemistry to develop several diversity-oriented fluorescence libraries (DOFL) and successfully applied them to the discovery of imaging probes for a number of biological targets.^[5] Among our libraries is a rosamine library synthesized using solid-phase chemistry^[6] to provide more flexibility within the rhodamine scaffold, which has excellent photophysical properties. By screening this library against a muscle-formation cell culture, we previously discovered a compound that controls muscle differentiation.^[7] To additionally evaluate the application of rosamine derivatives as stem-cell-selective probes, we have screened the library against embryonic stem cells (ESC) in this study and discovered a novel fluorescent compound, called the compound of designation yellow 1 (CDy1, $\lambda_{ex}/\lambda_{em} = 535/570$ nm), that selectively stains ESC and iPSC as well.

For high-throughput screening, we incubated mouse ESC (mESC) and mouse embryonic fibroblast (MEF) feeder cells with 280 rosamine compounds at a concentration of 500 nM in 384-well microplates. After 0.5, 24, and 48 hours, tetramethylrhodamine isothiocyanate (TRITC) fluorescence and bright-field images were taken using an ImageXpress^{MICRO} imaging system. From this primary screening, 20 compounds that stained mESC consistently with stronger intensity than MEF were manually selected. As a secondary screening, we incubated mESC and MEF separately with each of the hit compounds and analyzed them using flow cytometry and

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found CDy1 to be the most selective for mESCs among the 20 hit compounds (Figure 1 a–c; see Schemes S1 and S2 in the Supporting Information). For a more systematic structure–selectivity relationship study, we synthesized a number of

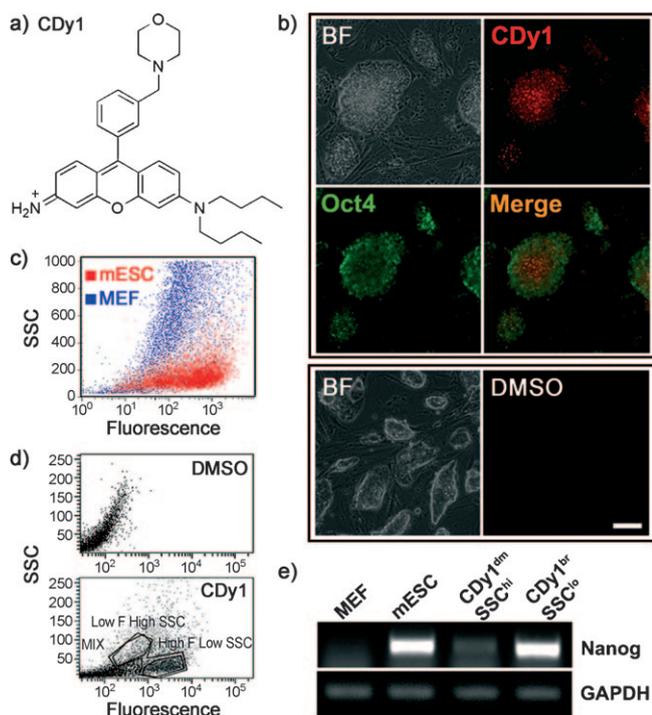


Figure 1. Selective staining of mESC by CDy1. a) Chemical structure of CDy1. b) Upper panel: CDy1-stained mESC were immunostained with anti-Oct4 antibody; lower panel: DMSO was used as a negative control. c) Flow cytometry dot-plot image of CDy1-stained mESC and MEF. Images of pure cell populations were overlaid. d) Flow cytometry dot-plot image of mESC and MEF mixture. Upper panel: mESC and MEF mixed cells incubated with DMSO; lower panel: mESC and MEF mixed cells incubated with CDy1. e) Nanog gene expression analysis using RT-PCR. SSC^{low} CDy1^{bright} and SSC^{high} CDy1^{dim} cells were sorted from a mESC and MEF mixture after CDy1 staining. BF = bright field; scale bar: 100 μm .

CDy1 analogues by modifying the morpholine group (see Scheme S1 in the Supporting Information) because most of the 20 primary hits had a di-*n*-butyl group in common. The mESC selectivity of all the analogues were, however, much lower than CDy1 which showed 12.2-fold higher intensity in mESC than in MEF. This result suggests that the morpholine group is also important for ESC selectivity of CDy1 (see Table S1 and Figure S3 in the supporting Information).

To evaluate the capability of CDy1 to isolate ESC from a mixed-cell population, we stained the MEF and mESC mixture with CDy1, gated the mixed cells into side scatter (SSC^{low} CDy1^{bright} and SSC^{high} CDy1^{dim}) regions, and collected 40 000 cells using a fluorescence-activated cell sorter (FACS) from each gate for analysis of a stem-cell marker Nanog gene expression and a colony-forming assay. The dot-plot image of the CDy1-stained mixed cells was similar to the overlay image of pure populations, whereas the cells incubated with dimethyl sulfoxide, used as a control, were not distinguishable

(Figure 1 d). RT-PCR analysis clearly showed that Nanog gene expression in SSC^{low} CDy1^{bright} cells is much higher than that in SSC^{high} CDy1^{dim} cells (Figure 1 e), and the numbers of colonies counted after a three-day culture were 604 and 6, respectively. We additionally induced differentiation of these CDy1-stained mESC by culturing in media without the leukemia inhibitory factor (LIF). After two weeks of differentiation, the expression of ectoderm, mesoderm, and endoderm markers were verified by immunocytochemistry (see Figure S4 in the Supporting Information). These data demonstrate that CDy1 can be used for mESC enrichment from a mixed-cell population without affecting the properties of the stem cells.

Having found that CDy1 selectively stains ESC, we applied the dye to iPSC which was generated from MEF of transgenic mice that express green fluorescent protein (GFP) under the control of the Oct4 (also known as Pou5f1) promoter. The reprogramming was performed in a 6-well culture dish by retroviral introduction of four transcription factors, Oct4, Sox2, Klf4, and c-Myc,^[8] and iPSC generation was verified by GFP expression, an alkaline phosphatase assay, and immunostaining of SSEA-1 at 17 days post infection (dpi). We found CDy1 also selectively stains the iPSC colony (see Figure S5 in the Supporting Information). When the 155 colonies grown in a 6-well plate cells were treated with CDy1 at 17 dpi, 101 colonies (65%) were both CDy1 and GFP positive, 26 (17%) were CDy1-only positive, 4 (3%) were GFP-only positive and 24 (15%) were negative for both CDy1 and GFP, despite the fact that the morphology of the colonies was indistinguishable (see Figure S6 in the Supporting Information). In a cell culture treated with CDy1 at an earlier time point of iPSC generation (10 dpi), an increasing number of CDy1-stained colonies started to show a GFP signal (Figure 2 a). To perform a more systematic analysis, we stained the cells with CDy1 at 2 dpi, when iPSC was not distinguishable by any means, and tracked the CDy1 and GFP signals by daily acquisition of cell images using an ImageXpress^{MICRO} system. At 10 dpi, when small colonies started to appear, we selected 342 CDy1-positive colonies that were not expressing GFP and monitored their GFP expression up to 25 dpi. More and more colonies started to express GFP during this period, and 338 (99%) out of the 342 tracked colonies were GFP positive at 25 dpi (Figure 2 b). During this period any detectable differences in the number of GFP-positive colonies or cell morphology were not observed compared to untreated iPSC. In contrast, we induced mESC differentiation by removing the LIF from the culture media and observed some cells that were morphologically distinguishable from mESC 3 days later. Most of the differentiated cells were not stained by CDy1, whereas some other cells having mESC morphology were stained by the dye, and showed similar patterns for immunocytochemical staining using the Oct4 antibody (Figure 3). This result was additionally confirmed in lineage-specific cells differentiated from mESC by embryoid body formation (see Figure S4 in the Supporting Information).

In our previous study with the rosamine library,^[7] CDy1 was among the compounds targeting mitochondria. To examine if CDy1 localizes in mitochondria in mESC, we co-

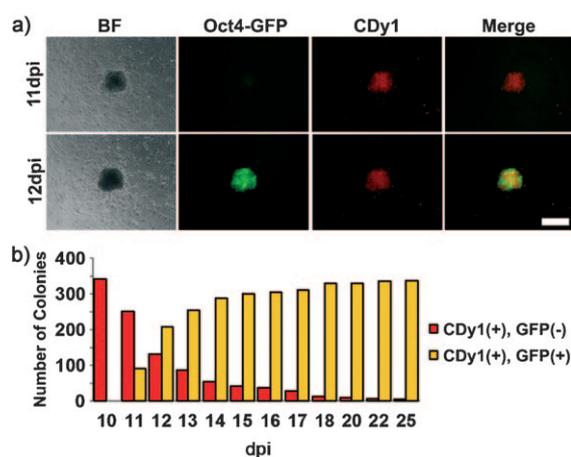


Figure 2. iPSC staining by CDy1 at an early stage of reprogramming. a) iPSC selective staining by CDy1. At 10 days post retroviral infection (dpi) with Oct4, Sox2, Klf4, and c-Myc, the iPSCs generated from Oct4-GFP transgenic mouse MEF were stained with CDy1. The pictures of the same colony were taken at 11 and 12 dpi. b) Time-course analysis of CDy1-stained colonies. Among the 342 CDy1-positive colonies that were not expressing GFP counted at 10 dpi, 338 colonies expressed GFP at 25 dpi. Scale bar: 200 μ m

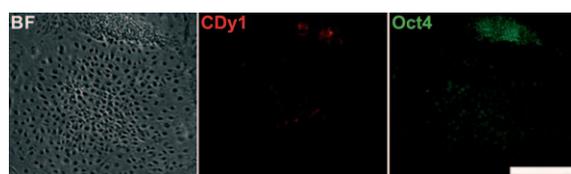


Figure 3. Differentiated mESC staining with CDy1. Morphologically distinguishable differentiated cells were observed after a three-day culture of mESC in the absence of LIF. Most of those cells were CDy1 negative, whereas some other cells that retained mESC morphology were stained by the dye. Immunocytochemistry with the Oct4 antibody performed on the following day showed a similar pattern of staining CDy1. Scale bar: 100 μ m.

stained the cells with CDy1 and a mitochondria-staining commercial dye (MitoTracker Deep Red 633) and observed a CDy1 staining pattern that was very similar to that of the MitoTracker staining (Figure 4). In addition to the mitochondrial membrane potential which sequesters many cationic rhodamine and rosamine compounds, other factors such as stem-cell-specific proteins appear to play roles in the entry and retainment of CDy1, rendering it stem-cell selective. A more detailed mechanism remains to be elucidated.

Among the few fluorescent dyes used for stem-cell staining^[9] is Aldefluor, which employs a fluorescent substrate BODIPY-aminoacetaldehyde for aldehyde dehydrogenase (ALDH)1A1.^[10] It has been used to identify and isolate certain types of stem cells including hematopoietic, neural and mammary stem cells as well as cancer stem cells. Because whether or not Aldefluor stains ESC has not been known, we compared the cell selectivity of CDy1 with Aldefluor and observed that Aldefluor stains neither mESC nor a human ESC (hESC). Reciprocally, CDy1 stained both mESC and hESC but not the human lung cancer cell line H522 which is

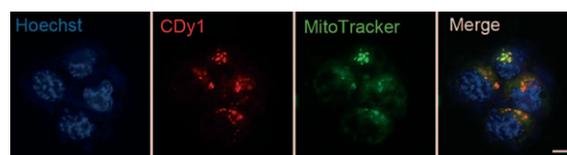


Figure 4. Co-localization of CDy1 and MitoTracker. mESC were incubated with 500 nM CDy1, 200 nM MitoTracker Deep Red 633, and 4 μ g mL⁻¹ Hoechst for 30 min at 37°C, and then washed with PBS (pH 7.4) two times. The cells were mounted on a glass slide and the images were recorded using a Nikon ECLIPSE Ti fluorescence microscope equipped with a \times 100 objective lens. PBS=phosphate-buffered saline. Pearson's correlation coefficient=0.88; scale bar: 5 μ m.

known to express a high level of ALDH1A1 and is stained by Aldefluor^[11] (Figure 5). The stemness of hESC BG01V used in this study was verified by immunocytochemical staining of TRA-1-60 (see Figure S7 in the Supporting Information).

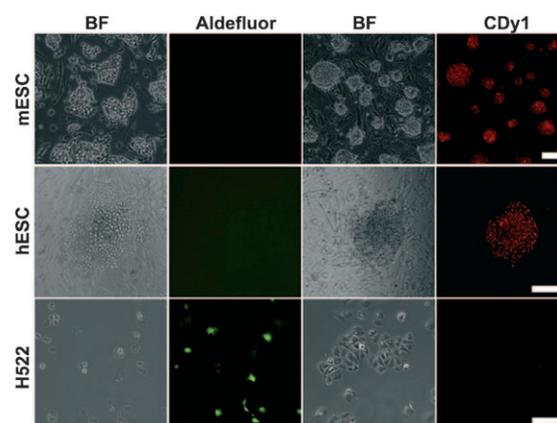


Figure 5. Comparison of CDy1 and Aldefluor staining in mESC and hESC (BG01 V). Aldefluor does not stain ESC, whereas CDy1 stains ESC but not the human lung cancer cell line H522, which expresses ALDH1a1. The cells cultured on a six-well plate were incubated with 1 μ M Aldefluor for 1 h at 37°C and then washed with PBS three times before the Aldefluor assay buffer was added. Bright-field and fluorescence (FITC filter for Aldefluor and TRITC filter for CDy1) images were recorded using a Nikon ECLIPSE Ti fluorescence microscope. FITC=fluorescein isothiocyanate. Scale bars: 100 μ m.

In summary, we have developed a novel bioimaging probe, CDy1, for ESC and iPSC detection. The experimental results presented herein strongly demonstrate that CDy1 can be used for the identification and isolation of live ESC and iPSC without the aid of a genetic reporter system at an earlier stage of the reprogramming and during the ESC differentiation. To our knowledge, no ESC- or iPSC-selective fluorescent probe has been reported yet. This new probe, CDy1, would be a useful tool for stem-cell research.

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