HHS Public Access

Author manuscript

Nat Commun. Author manuscript; available in PMC 2014 October 23.

Published in final edited form as:

Nat Commun. ; 5: 3662. doi:10.1038/ncomms4662.

A red-shifted fluorescent substrate for aldehyde dehydrogenase

Il Minn[1,*], **Haofan Wang**[1,*], **Ronnie C. Mease**[1], **Youngjoo Byun**[4], **Xing Yang**[1], **Julia Wang**[2], **Steven D. Leach**[2], and **Martin G. Pomper**[1,3]

[1]Russel H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University, Baltimore, MD.

[2]Department of Surgery and the McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD

[3]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD.

[4]College of Pharmacy, Korea University 2511 Sejong-ro, Jochiwon-eup, Yeongi-gun, Chungnam 339-700, South Korea.

Abstract

Selection of cells positive for aldehyde dehydrogenase (ALDH) activity from a green fluorescent background is difficult with existing reagents. Here we report a red-shifted fluorescent substrate for ALDH, AldeRed 588-A, for labeling viable ALDH^{pos} cells. We demonstrate that AldeRed 588-A successfully isolates ALDH^{hi} human hematopoietic stem cells from heterogeneous cord blood mononuclear cells. AldeRed 588-A can be used for multi-color applications to fractionate ALDH^{pos} cells in the presence of green fluorophores including the ALDEFLUOR™ reagent and cells expressing eGFP. AldeRed 588-A stains ALDH^{pos} murine pancreatic centroacinar and terminal duct cells, as visualized by fluorescent microscopy. AldeRed588-A provides a useful tool to select stem cells or study ALDH within a green fluorescent background.

Introduction

Aldehyde dehydrogenase (ALDH) is an evolutionarily conserved enzyme with pyridine nucleotide dependent oxidoreductase activity that performs a variety of critical cellular processes[1]. These include production of retinoic acid essential for mammalian development[2], metabolism of fats and amino acids, and detoxification of endogenous and exogenous sources of hazardous aldehyde byproducts[3]. Twenty human *ALDH* genes have

Corresponding Author: Martin G. Pomper, M.D., Ph.D., Johns Hopkins Medical School, 1550 Orleans Street, 492 CRB II, Baltimore, MD 21287-0014, Ph: 410-955-2789, Fax: 443-817-0990, mpomper@jhmi.edu.

*Equal contribution.

Authorship Contributions

M.G.P and S.D.L conceived the project. I.M. designed and performed all *in vitro* validation of candidate ALDH substrates and works related to the TNR mice and human cord blood cells. R.C.M., Y.B., and H.W. designed the synthesis of candidate ALDH substrates. H.W. synthesized candidate ALDH substrates. X.W. isolated and characterized AldeRed 588-A. J.W. performed the isolation of CA/TD cells and imaging. I.M., R.C.M. and M.G.P. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

been identified and many of their functions are still unknown[4]. For the past two decades ALDH has been studied as a potential universal marker for normal and cancer stem cells, as certain isoenzymes of the ALDH superfamily have been identified as key elements of these cells[5]. For example, Aldh1a1 and Aldh3a1 have been implicated in the protection of stem cells from cytotoxic drugs. ALDH^{pos} stem cells have been used as resources for regenerative medicine in preclinical models[6] and in an ongoing clinical trial for ischemic cardiomyopathy (clinicaltrial.gov, NCT00314366). ALDH1 has been identified as a marker used to isolate cancer stem cells of various human malignancies including bladder, breast, cervical, colon, head and neck, liver, lung, pancreas, prostate, and ovary [5]. Recently Gerber *et al*. showed that the presence of leukemic stem cells with intermediate ALDH activity (ALDH^{int}) could be used as a predictor for relapse after therapy, whereas normal hematopoietic stem cells (HSCs) retain high ALDH activity[7]. Since these normal and cancer stem cells are very rare, methods to identify and isolate viable, functionally active ALDH^{pos} cells are needed to characterize or utilize them.

The ALDEFLUOR™ reagent (Aldagen Inc., Durham, NC) has enabled the primary commercial assay used today for isolation of viable ALDH^{pos} cells, which was patterned after the original dansyl aminoacetaldehyde (DAAA) based assay developed by Jones *et al*[8]. Although very sensitive and specific for staining viable ALDH^{Pos} cells, because it emits in the green region of the electromagnetic spectrum (512 nm), the ALDEFLUOR™ reagent cannot be simultaneously utilized in cells or mice expressing green fluorescent proteins[9,10]. This has limited the use of many valuable cell and animal models with green fluorescent signals to study ALDH, and there have been increasing demands for non-green functional ALDH markers. Here we report the synthesis, *in vitro* validation, and applications of a red-shifted fluorescent substrate of ALDH.

Results

Syntheses of candidate aldehyde dehydrogenase substrates

We synthesized three candidate substrates of aldehyde dehydrogenase (ALDH) containing fluorophores that emit in the red region of the spectrum (Fig. 1). Three red fluorophores, (*E*)-4-(4-(dimethylamino)styryl)-1-(3-isothiocyanatopropyl)pyridin-1-ium chloride **1**, BODIPY® 576/589 succinimidyl ester **2**, and BODIPY® 650/665 succinimidyl ester **3** were conjugated to aminoacetaldehyde diethyl acetal to produce diethyl acetals **4**–**6**, which were purified by reverse phase liquid chromatography or flash column chromatography. Maximum excitation and emission wavelengths for **4**, **5**, and **6** were 493/590 nm, 588/599 nm, and 659/671 nm, respectively (Supplementary Fig. 1). Acid hydrolysis of the diacetals to produce the corresponding aldehydes, AldeRed 493-A, AldeRed 588-A, and AldeRed 659-A was performed immediately before the assay.

In vitro validation of candidate ALDH substrates

We tested the ALDH specificity of the three candidate substrates using human and murine cell lines that express different levels of ALDH, namely, K562 (ALDH^{hi}), L1210 (ALDH^{low}), and L1210/cpa (ALDH^{hi})[11]. We analyzed cell uptake and retention of the substrates in the absence and presence of the ALDH inhibitor, diethylaminobenzaldehyde

(DEAB)[8]. As with the original ALDEFLUOR™ reagent, it is the acid-deprotected aldehyde form of the candidate substrate that diffuses into cells and is converted into the corresponding carboxylate by ALDH, which is retained. Substrates were evaluated using the LSR II (BD Biosciences, San Jose, CA) fluorescence-activated cell sorter (FACS) equipped with four lasers and 14 emission filters (**Methods** and Supplementary Figs. 2–4). Of the three compounds tested AldeRed 588-A demonstrated specific uptake for both K562 and L1210/cpa cells when compared with the DEAB-treated control, indicating ALDH substrate specificity (Fig. 2a). All three substrate candidates stained cells as indicated by shifted signals in selected emission filter sets compared with unstained cells (Supplementary Figs. 2–4). However, AldeRed 493-A did not demonstrate increased signals for either $ALDH^{pos}$ cell line, and AldeRed 659-A exhibited only a minimal shift of fluorescent uptake in comparison with DEAB-treated control (Fig. 2a and Supplementary Figs. 2–4). We were able to use the basic analytical FACS device, FACSCalibur (BD Biosciences, San Jose, CA), with its single blue laser, to detect the cellular uptake of AldeRed 588-A using the FL2 filter (Supplementary Fig. 5). To examine further AldeRed 588-A as a substrate for ALDH we compared the ability of the ALDEFLUOR™ reagent and AldeRed 588-A for detecting different levels of ALDH expression. We stained L1210 ($ALDH^{low}$) and L1210/cpa ($ALDH^{hi}$) cells and found that both reagents were able to differentiate these two cell lines (Fig. 2b). As further confirmation we performed co-staining with the ALDEFLUOR™ reagent and AldeRed 588-A. Both substrates proportionately co-stained $ALDH^{hi}$ K562 and L1210/cpa cell lines (Fig. 2c). Importantly, these data demonstrate that red fluorescent AldeRed 588-A could be used for co-staining with green fluorophores.

Isolation of $ALDH^{hi}$ human hematopoietic stem cells

Primitive adult stem cells are rare, suggesting that a single-step isolation method would be useful to maximize the efficiency of purification and minimize damage to cells. We tested AldeRed 588-A for its capacity to isolate $ALDH^{hi}$ stem cells from a heterogeneous mixture of cells in human cord blood. We labeled mononuclear cells derived from human cord blood with ALDEFLUOR™ and isolated the $ALDH^{hi}$ cell population that is not present in cells treated with DEAB (control) (Fig. 3a). Isolated cells were capable of giving rise to multiple types of differentiated colonies including BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM (Table 1 and Supplementary Fig. 6) indicating that these $ALDH^{hi}$ cells are enriched HSCs. We asked whether AldeRed 588-A could perform the same task as ALDEFLUOR™. AldeRed 588-A successfully isolated $ALDH^{hi}$ populations (Fig. 3b), which gave rise to differentiated colonies in a very similar pattern to cells isolated by ALDEFLUOR™ (Table 1 and Supplementary Fig. 6). We then co-labeled human cord blood mononuclear cells with ALDEFLUOR™ and AldeRed 588-A to determine whether both agents were staining the same HSC populations. We confirmed that cells labeled green and red represented identical $ALDH^{hi}$ populations that could be isolated by gating them into their respective channels by FACS (Fig. 3c, d). In addition, cells exhibited proportional staining patterns for both reagents indicating specificity of the staining (Supplementary Fig. 7). These results indicate that AldeRed 588-A can efficiently stain and isolate $ALDH^{hi}$ HSCs among heterogeneous cell populations and can be used with green ALDEFLUOR™ to increase the purity of $ALDH^{pos}$ cells, if necessary.

Fractionation of ALDH^{pos} cells from mice expressing eGFP

Since the BODIPY® fluorophore for the ALDEFLUOR™ reagent emits green light, cells harvested from transgenic mice expressing green fluorescent tags cannot be studied with this standard reagent. To test if one could use AldeRed 588-A with cells from a transgenic mouse expressing an enhanced green fluorescent protein (eGFP) tag, we attempted to enrich ALDH^{int} murine hematopoietic stem and progenitor cells (HSPCs) among eGFP-expressing bone marrow (BM) cells isolated from transgenic notch reporter [*TNR, Tg(Cp-EGFP)25Gaia*] mice[10]. The TNR mice express eGFP in cells with active notch signaling attributed to the C promoter (Cp) binding factor 1 (CBF1) binding site upstream of the eGFP gene. We chose to study the TNR mice for two reasons, both related to biologically relevant systems to assess the utility of our reagent. First, although it is known that murine HSPCs demonstrate high notch signaling activity [10], the function of notch signaling in maintenance of HSPCs is controversial [12,13]. Second, there has been disagreement with respect to using ALDH as a marker for murine HSPCs. While the ALDEFLUOR™ reagent has been used to isolate functional murine HSPCs [14,15], one group reported that *Aldh1a1* deficient mice had normal hematopoiesis and functional HSPCs [16]. The TNR mice provide a suitable model to test red fluorescent AldeRed 588-A for its ability to fractionate cells according to ALDH activity from eGFP^{pos} notch active cells. We isolated BM cells from 5- to 6-week old TNR mice and stained them with AldeRed 588-A. We fractionated those cells into 5 different populations: eGFP^{neg}/ALDH^{hi} **1**, eGFP^{pos}/ALDH^{hi} **2**, eGFP^{neg}/ALDH^{int} **3**, eGFP^{pos}/ALDH^{int} **4**, and eGFP^{neg}/ALDH^{lo} **5** (Fig. 4b). To examine in which population HSPCs reside, we isolated each population and performed colony-forming cell assays. We found that the eGFP^{pos}/ALDH^{int} cell population **4** formed colonies CFU-GEMM, CFU-GM, CFU-M, and BFU-E, while none of the other cell populations formed a single colony (Fig. 4e). To verify that HSPCs reside within the eGFP^{pos}/ALDH^{int} population we performed multi-color FACS analysis for lineage^{-}/c-Kit^{+}/Sca1^{+} (LKS) HSPCs in addition to ALDH staining using AldeRed 588-A. LKS cells were present within the eGFP^{pos}/ALDH^{int} population **4** (Fig. 4c). Additionally, the red fluorescence of the LKS cells shifted when compared with DEAB-treated control, indicating that LKS cells possess active ALDH (Fig. 4d).

Staining of ALDH^{pos} murine pancreatic stem cells

As an additional example of the utility of AldeRed 588-A, we tested its capacity for identifying ALDH^{pos} cells *in vitro* with fluorescence microscopy. We have previously shown that a subset of murine pancreatic centroacinar and terminal duct (CA/TD) cells expressing abundant Aldh1a1 and Aldh1a7 can be imaged and isolated using the ALDEFLUOR™ reagent[17]. We have also shown that the isolated CA/TD cells exhibited characteristics of murine pancreatic progenitor cells. Using AldeRed 588-A and the ALDEFLUOR™ reagent we stained isolated murine CA/TD cells and examined them under fluorescence microscopy. Both red and green substrates successfully stained CA/TD cells without overlap between the detection filters for each reagent (Fig. 5a, b). Upon co-staining, red and green signals overlapped, indicating ALDH specificity (Fig. 5c and Supplementary Fig. 8).

Discussion

Stem cells are important new reagents in biomedical research. They can provide experimental models for target discovery[18] or toxicity testing[19], and can be used as tools for screening drugs and in developing therapies for a host of disorders[20,21]. Stem cells can provide a direct source of materials for regenerative medicine[22]. The ability to prepare pure, undamaged and functionally active stem cells is the critical first step for all such applications. Isolation of stem cells is generally done by selection with monoclonal antibodies that recognize stem cell-specific cell surface markers or by utilizing functional markers of stem cell activity, such as elevated expression of multi-drug efflux pumps[23] and ALDH[8]. Combinations of multiple markers, often requiring multiple steps of selection, are used to isolate rare stem cells from heterogeneous cell populations. A brief, single-step isolation method would be preferred to minimize loss of and damage to rare stem cells. We present a red-shifted agent that provides additional flexibility for utilizing ALDH as a marker for stem cell isolation. The ALDEFLUOR™ assay, the only commercially available method to detect and isolate functionally active $ALDH^{pos}$ cells, depends on differences in fluorescence intensity obtained for the cellular retention of green fluorescent substrate in the presence and absence of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). As a red-shifted substrate for ALDH, AldeRed 588-A will enable isolation of stem cells from a green fluorescent background, which is increasingly prevalent as many genes – including those in transgenic models – are tagged with eGFP. In addition, since many cells demonstrate autofluorescence in the green region of the spectrum[24], there is a chance of false positive results from stem cell isolation with ALDEFLUOR™. AldeRed 588-A could provide an additional way to confirm the purity of ALDH-active cells when used in conjunction with ALDEFLUOR™.

In order to act as a functional probe for ALDH, a compound should possess three characteristics: an aldehyde moiety that can serve as a substrate for ALDH; suitable hydrophobicity for free diffusion into cells; and capacity for subsequent trapping within the cytoplasm after conversion of the aldehyde into the corresponding acid by ALDH. Our initial choice for three red fluorophores was based on their lack of charged groups, such as sulfonic acid or carboxylic acid, found in other commercial dyes such as the Cy series, Alexa Fluor® or IRDye®. Those charged groups may prevent the substrate from penetrating the plasma membrane, limiting access to cytoplasmic ALDH. Of the three compounds tested only AldeRed 588-A met all three criteria. The degree of trapping is particularly challenging to control as those two compounds initially stained cells but failed to accumulate. Vaidyanathan *et al.* reported two radiolabeled ALDH substrates that could be converted into the acid form by the purified enzyme[25]. Both substrates, however, failed to accumulate inside of cells with high ALDH activity when compared with the DEAB control. The authors discussed the lack of appropriate physical characteristics (hydrophobicity) as a possible cause.

AldeRed 588-A has a red-shifted emission spectrum and possesses physical properties that enable it to function as a probe for ALDH *in vivo*. Although AldeRed 588-A would provide a tool to researchers using green fluorescent materials, it has a limitation for multi-color application, e.g. 14 color sorting with flow cytometry, due to its broad emission spectrum

(Supplementary Figs. 2–4). AldeRed 588-A would, however, allow for six color sorting with the LSR II (BD Biosciences, San Jose, CA) FACS equipped with four lasers and 14 emission filters. Nevertheless, we continue to search for a fluorescent substrate for ALDH with a narrow emission spectrum to enable enhanced multi-color sorting.

We initially chose cell lines known to express abundant, functional ALDH homogeneously for cell-based validation of our candidate substrates (Fig. 2). We then tested whether AldeRed 588-A could isolate ALDH^{hi} cells among a mixture of heterogeneous cell populations using mononuclear cells from human cord blood. Human hematopoietic stem cells are known to express high levels of ALDH, and the ALDEFLUOR™ reagent has been used to purify ALDH^{hi} HSCs *via* simple staining and sorting[8,26]. AldeRed 588-A demonstrated essentially the same efficiency as ALDEFLUOR™ for isolating ALDH^{hi} HSCs and can be used simultaneously (Fig. 3c, d, Fig. 5c, Supplementary Fig. 6, and Supplementary Fig. 8). Such co-staining could be used to increase further the specificity of the detection of true ALDH^{pos} cells, particularly in the context of a green fluorescent background.

It has been reported that Aldh1a1 is unnecessary for murine hematopoietic stem and progenitor cell (HSPC) function but also that *Aldh1a1*−/− BM cells stain positive with the ALDEFLUOR™ reagent, confusing the issue of ALDH function in HSPCs[16]. Recently Garaycoechea *et al*. demonstrated that murine HSPCs could be stained using ALDEFLUOR™ by virtue of the activity of ALDH2, which plays a role in detoxifying acetaldehyde[27]. Our results suggest that murine HSPCs possess ALDH activity. It will be interesting to examine whether AldeRed 588-A exhibits a similar staining pattern to ALDEFLUOR™ with respect to HSPCs of *Aldh1a1*−/− and *Aldh2*−/− mice. We anticipate that AldeRed 588-A will provide increased flexibility to study a variety of different aspects of ALDH, particularly in the presence of a green fluorescent background.

Methods

Cell Lines and animals

The K562 human chronic myelogenous leukemia cell line was purchased from American Type Culture Collection (CLL-243™) and maintained in suspension in IMDM media supplemented with 10% FBS. The murine leukemia cell line L1210 (ALDH^{low}) and L1210/cpa (ALDH^{hi}) were provided by Dr. Richard J. Jones (Johns Hopkins University) and maintained in suspension in RPMI 1640 supplemented with 10% FBS. All cells were grown at 37°C in a humidified incubator with 5% CO_2. All animal experiments were performed in accordance with protocols approved by Johns Hopkins University Animal Care and Use Committee (ACUC).

Reagents and analyses

BODIPY® 576/589 succinimidyl ester and BODIPY® 650/665 succinimidyl ester were purchased from Life Technologies Inc., (Grand Island, NY). (*E*)-4-(4-(dimethylamino)styryl)-1-(3-isothiocyanatopropyl)pyridin-1-ium chloride **1** was synthesized analogously to the previously reported di-butyl analog[28]. Chemicals and solvents for

synthesis and high-performance liquid chromatography (HPLC) were acquired from Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA), and used without further purification. HPLC was performed on an Agilent Prostar System equipped with a 325 Variable wavelength detector and controlled by Galaxie Software using a Phenomenex 4.6 × 250 mm Luna C18, 10 micron column. AldeRed 493 aldehyde diethyl acetal (Supplementary Fig. 9a, b) and AldeRed 493-A (Supplementary Fig. 9c, d) were chromatographed using Method 1. AldeRed 588 aldehyde diethyl acetal (Supplementary Fig. 10a, b), AldeRed 588-A (Supplementary Fig. 10c, d), AldeRed 659 aldehyde diethyl acetal (Supplementary Fig. 11a, b), AldeRed 659-A (Supplementary Fig. 11c, d), BODIPY™-aminoacetaldehyde-diethyl acetate (Supplementary Fig. 12a, b), and activated ALDEFLUOR™ Reagent (Supplementary Fig. 12c, d) were chromatographed using Method 2. Method 1: Solvent A: 100% water; Solvent B: 100% methanol; 0–5min 100% A, 5–25 min linear gradient from 100% A to 100% B, 25–30 min 100% B, 30–35 min linear gradient from 100% B to 100% A, 35–38 min 100% A, 2 mL/min. Method 2: Solvent A: 100% water; Solvent B: 100% acetonitrile; 0–20 min linear gradient from 90% A/10% B to 10% A/90% B; 20–25min 10% A/90% B, 25–30 min linear gradient from 10% A/90% B to 90% A/10% B, 2 mL/min. ^{1}H-NMR spectra were obtained on a Bruker Avance 400 MHz Spectrometer. High resolution mass spectra were obtained by the University of Notre Dame Mass Spectrometry & Proteomics Facility (Notre Dame, IN) using ESI either by direct infusion on a Bruker micrOTOF-II or by LC elution *via* an ultra-high pressure Dionex RSLC with C18 column coupled with a Bruker micrOTOF-Q II. The excitation and emission profiles of synthesized products were measured using a spectrofluorophotometer (Shimadzu, RF-5301pc, Columbia, MD).

Synthesis of AldeRed 493 amino acetal aldehyde diethylacetal 4

Aminoacetaldehyde diethyl acetal (0.024 mmol, 3 μL) was added to a solution of (*E*)-4-(4-(dimethylamino)styryl)-1-(3-isothiocyanatopropyl)pyridin-1-ium chloride **1** (3.6 mg, 0.01 mmol in 1 mL of DMF) and 5 μL of triethylamine and mixed by stirring for 30 min in the dark. 5 mL of water was added and the resulting solution was extracted with ether (2 × 5 mL). Water in the aqueous phase was evaporated under vacuum and the residue was purified by passing through a small C18 column, eluted with 1:1 water/acetonitrile. Yield: 1.8 mg, 0.004 mmol, 40%. ^{1}H NMR(400MHz, DMSO-d_6): δ=1.10(t, 6H, J=7.6Hz); 2.09–2.13(m, 2H); 3.0(s, 6H); 3.4–3.5(m, 6H); 3.55–3.62(m, 2H); 4.43(t, 2H, J=6.8Hz); 4.5–4.6(m, 1H); 6.77(d, 2H, J=8.8Hz); 7.15(d, 1H, J=16.4Hz); 7.58(d, 2H, J=8.4Hz); 7.90(d, 1H, J=15.6Hz); 8.04(d, 2H, J=6.8Hz); 8.74(d, 2H, 6.8Hz). HRESI-MS $C_{25}H_{37}N_4O_2S^+$ calcd 457.2632; found 457.2632 (Supplementary Fig. 13).

Synthesis of AldeRed 588 aldehyde diethyl acetal 5

Aminoacetaldehyde diethyl acetal (0.024 mmol, 3 μL) was added to BODIPY® 576/589 succinimidyl ester **2** (5 mg, 0.012 mmol in 1 mL of THF) and mixed by stirring for 30 min in the dark. After evaporating THF, AldeRed 588 aldehyde diethyl acetal **5** was purified by flash column chromatography using ethyl acetate/hexane (1:1) as an eluent to give 3.8 mg, 0.0086 mmol 75% yield. ^{1}H NMR(400MHz, $CDCl_3$): δ=1.16(t, 6H, J=7Hz); 2.65(t, 2H, J=7.2Hz); 3.31(t, 2H, J=7.2Hz); 3.37(t, 2H, J=5.6Hz); 3.48(m, 2H); 3.65(m,2H); 4.43(t, 1H, 5.6Hz); 5.82(br, 1H); 6.27(d, 1H, 3.6=3.6Hz); 6.36(br, 1H); 6.81(d, 1H, J=4Hz); 6.85(d, 1H,

4.4Hz); 6.96(s, 1H); 6.97(s, 1H), 7.02(d, 1H, J=4.4Hz); 7.16(s, 1H). HRESI-MS $C_{22}H_{28}BF_2N_4O_3$ calcd 445.4222; found 445.4222 (Supplementary Fig. 13).

Synthesis of AldeRed 659 aldehyde diethyl acetal 6

Aminoacetaldehyde diethyl acetal (0.016 mmol, 2 μL) was added to BODIPY® 650/665 succinimidyl ester **3** (5 mg, 0.0078 mmol in 1 mL of THF) and mixed by stirring for 30 min in the dark. After evaporating THF, AldeRed 659 aldehyde diethyl acetal **6** was purified by flash column chromatography using ethyl acetate/hexane (1:1) as an eluent to give 3.5 mg, 0.0053 mmol, 70% yield. ^{1}H NMR(400MHz, $CDCl_3$): δ=1.19(t, 6H, J=7.8Hz); 1.33–1.37(m, 2H), 1.5–1.58(m, 2H); 1.6–1.68(m, 2H); 2.16(t, 2H, J=7.8Hz); 3.31–3.38(m, 4H); 3.46–3.55(m, 2H); 3.62–3.71(m, 2H); 4.46(t, 1H, J=5.2Hz); 4.51(s, 2H); 5.65(br, 1H); 6.36–6.39(m, 1H); 6.57–6.62(m, 1H); 6.83–6.88(m, 2H); 6.89(d, 1H, J=4Hz); 6.92–6.98(m, 4H); 7.0(d, 1H, J=4Hz); 7.18(m, 1H); 7.50–7.58(m, 3H). HRESI-MS $C_{35}H_{43}BF_2N_5O_5$ calcd 662.3326; found: 662.3326 (Supplementary Fig. 13).

Preparation of aldehyde derivatives

All diethyl acetal precursors to the final aldehyde derivatives were dissolved in 100% DMSO at 5 mM and stored at −20°C in stock solutions. Immediately before the assay a 25 μL aliquot of each stock solution was deprotected by adding the same volume of 2N HCl followed by incubation for 30 min at room temperature. The resulting reaction mixtures were neutralized by adding 350 μL of assay buffer (PBS supplemented with 1% FBS and 50 μM verapamil).

Isolation of AldeRed 588-A

In order to characterize AldeRed 588-A produced upon hydrolysis, AldeRed 588 aldehyde diethyl acetal **5** (0.5 mg in 300 μL) was mixed with 300 μL of 2N HCl and incubated for 30 min at room temperature. The reaction mixture was then diluted with 2 mL of water. The clear purple solution was injected into the HPLC. The purification of AldeRed-588A was performed using an Agilent 1260 infinity preparative HPLC system equipped with a Phenomenex Luna C18, 10 micron column and a flow rate of 10 mL/min. The desired product eluted at 3.3 min with acetonitrile/water (40/60) and collected. (The solvent front eluted at 1.1 min.) The collected fraction was frozen at −78°C immediately and lyophilized to dryness. Approximately 0.4 mg of AldeRed-588A was obtained as a dark powder. HRESI-MS $C_{18}H_{18}BF_2N_4O_2$ calcd 371.1485; found: 371.1489 (Supplementary Fig. 14).

In vitro fluorescence uptake assay for ALDH activity

One million cells were resuspended in assay buffer and 5 μL of the corresponding aldehyde (ALDEFLUOR™, AldeRed 493-A, AldeRed 588-A or AldeRed 659-A) was added to the suspension. In each case a 0.5 mL aliquot of the suspension was immediately added to a 5 mL polystyrene round bottom tube (BD Biosciences, San Jose, CA) containing 5 μL of diethylaminobenzaldehyde (DEAB) (STEMCELL Technologies, Inc., Vancouver, BC, Canada). Cells were incubated in a water bath at 37°C for 30 min followed by washing with 4 mL of cold assay buffer once. Cells were resuspended in cold assay buffer (5×10^5/200 μL) and stored on ice until analyzed. Stained cells were analyzed by FACS LSRII (BD

Biosciences, San Jose, CA) equipped with four lasers (405, 488, 532 and 633 nm) and 14 emission filters. All three candidate ALDH substrates were tested on all 15 filters. AldeRed 588-A was also tested on a FACSCalibur (BD Biosciences, San Jose, CA) with 488 nm blue laser and phycoerythrin (PE) filters.

Isolation of ALDH^{hi} human hematopoietic stem cells

Frozen human cord blood mononuclear cells were purchased from ALLCELLS (Alamenda, CA). Cells were thawed according to the provider's instructions immediately before use followed by re-suspension in assay buffer (1×10^6 cells/mL). The staining procedure for ALDEFLUOR™ and AldeRed 588-A was same as described above for the *in vitro* fluorescence uptake assay. ALDH^{hi} cells were sorted into RPMI supplemented with 10% FBS using FACSAria (BD Biosciences, San Jose, CA) equipped with four lasers (405, 488, 532 and 633 nm) and 14 emission filters. Five hundred ALDH^{hi} cells were plated in 1 mL of Methocult® H4435 (STEMCELL Technologies, Inc., Vancouver, BC, Canada) and incubated in a humidified incubator at 37°C with an atmosphere of 5% CO_2 for one week. Colonies formed were counted one week later and photographed using a Nikon TE-200 inverted microscope.

Enrichment of HSCs from bone marrow cells of TNR mice

Transgenic notch reporter (TNR) mice [*Tg(Cp-EGFP)25Gaia*] were provided by Dr. Shyam Biswal (Johns Hopkins School of Public Health). Bone marrow cells from sixweek- old male mice were harvested by repeatedly flushing the femur, tibia, and pelvis with a 26Gx5/8 needle. Red blood cells were depleted using RBC lysis buffer (BioLegend, San Diego, CA) according to the manufacturer's instructions. Cells were stained with AldeRed 588-A and sorted into five populations (eGFP^{neg}/ALDH^{pos}, eGFP^{neg}/ALDH^{int}, eGFP^{neg}/ALDH^{hi}, eGFP^{pos}/ALDH^{int}, and eGFP^{pos}/ALDH^{hi}) using FACS Aria (BD Biosciences, San Jose, CA) with fluorescein isothiocyanate (FITC) and PE-Texas Red filters for eGFP and AldeRed 588-A, respectively. Twenty thousand cells from each population were plated in 1 mL of Methocult® M3434 (STEMCELL Technologies, Inc., Vancouver, BC, Canada) and incubated in a humidified incubator at 37°C with and atmosphere of 5% CO_2 for one week. Colonies formed were counted one week later and photographed using a Nikon TE-200 inverted microscope. A mouse hematopoietic lineage cocktail (eFluor®450, eBiosciences, San Diego, CA) containing anti-mouse Ly-6A/E (PE-Cy7, BioLegend, San Diego, CA), and anti-mouse CD117 (APC-Cy7, BioLegend, San Diego, CA) was used according to the manufacturer's instruction to stain and isolate lineage$^-$/c-Kit$^+$/Sca1$^+$(LKS) cells using LSR II.

Microscopic ALDH staining of murine pancreatic cells

Mouse pancreatic acinar preparation was performed as previously described[29]. Briefly, adult CD1 mouse pancreas was harvested and digested in 1 mg/mL collagenase-P (Roche, Indianapolis, IN) at 37°C in a water bath for 15 min. The collagenase-P reaction was terminated by the addition of ice cold HBSS with 5% FBS. Following multiple washes, collagenase-digested pancreatic tissue was filtered through a 500 μm polypropylene mesh (Spectrum Laboratories, Rancho Dominguez, CA). The resulting acinar units were re-

suspended in 20 mL of ALDEFLUOR™ assay buffer (STEMCELL Technologies, Inc.). To label the cells with fluorescent agent, 5 μL of green ALDEFLUOR™ substrate and 5 μL of AldeRed 588-A were used per 1 mL of ALDEFLUOR™ assay buffer. Cell staining proceeded at 37°C for 50 min. A DEAB control reaction was performed in parallel. Samples were placed in ice to terminate the reaction. Live cell imaging was performed on a Nikon A1 Confocal system (Nikon Instruments, Inc., Melville, NY). The optical configuration was optimized for the DEAB control sample eliminating possible signal in green and red channels. All images were taken using the same optical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Authors would like to thank Dr. Crystal Dusich for the synthesis of **1**. We also thank (all from Johns Hopkins) Drs. Shyam Biswal and Jung-Hyun Kim for providing the TNR mice, Dr. Richard Jones for providing L1210/cpa and L1210 cell lines, Dr. Wojciech Lesniak for helping with excitation and emission profiling and Dr. Hao Zhang and Ada Tam for providing guidance with FACS analyses. This work was funded by Maryland Stem Cell Research Fund grants 2010-MSCRFF-0109-00 and 2012-MSCRFE-0211-00 and NIH DK56211, CA134675 and CA098252.

References

1. Marchitti SA, Brocker C, Stagos D, Vasiliou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol. 2008; 4:697–720. [PubMed: 18611112]
2. Niederreither K, Subbarayan V, Dolle P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet. 1999; 21:444–448. [PubMed: 10192400]
3. Vasiliou V, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. Drug Metab Rev. 2004; 36:279–299. [PubMed: 15237855]
4. Black WJ, et al. Human aldehyde dehydrogenase genes: alternatively spliced transcriptional variants and their suggested nomenclature. Pharmacogenet Genomics. 2009; 19:893–902. [PubMed: 19823103]
5. Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev. 2011; 7:292–306. [PubMed: 21103958]
6. Balber AE. Concise review: aldehyde dehydrogenase bright stem and progenitor cell populations from normal tissues: characteristics, activities, and emerging uses in regenerative medicine. Stem Cells. 2011; 29:570–575. [PubMed: 21308868]
7. Gerber JM, et al. A clinically relevant population of leukemic CD34(+)CD38(−) cells in acute myeloid leukemia. Blood. 2012; 119:3571–3577. doi:blood-2011-06-364182 [pii]10.1182/blood-2011-06-364182. [PubMed: 22262762]
8. Jones RJ, et al. Assessment of aldehyde dehydrogenase in viable cells. Blood. 1995; 85:2742–2746. [PubMed: 7742535]
9. Shima K, et al. Potential utility of eGFP-expressing NOG mice (NOG-EGFP) as a high purity cancer sampling system. J Exp Clin Cancer Res. 2012; 31:55. doi:1756-9966-31-55 [pii]10.1186/1756-9966-31-55. [PubMed: 22672897]
10. Duncan AW, et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol. 2005; 6:314–322. doi:ni1164 [pii]10.1038/ni1164. [PubMed: 15665828]
11. Hilton J. Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. Cancer Res. 1984; 44:5156–5160. [PubMed: 6488175]
12. Pajcini KV, Speck NA, Pear WS. Notch signaling in mammalian hematopoietic stem cells. Leukemia. 2011; 25:1525–1532. doi:10.1038/leu.2011.127leu2011127 [pii]. [PubMed: 21647159]

13. Bigas A, Espinosa L. Hematopoietic stem cells: to be or Notch to be. Blood. 2012; 119:3226–3235. doi:blood-2011-10-355826 [pii]10.1182/blood-2011-10-355826. [PubMed: 22308291]
14. Pearce DJ, Bonnet D. The combined use of Hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. Exp Hematol. 2007; 35:1437–1446. doi:S0301-472X(07)00353-0 [pii]10.1016/j.exphem.2007.06.002. [PubMed: 17656008]
15. Armstrong L, et al. Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. Stem Cells. 2004; 22:1142–1151. doi: 22/7/1142 [pii]10.1634/stemcells.2004-0170. [PubMed: 15579635]
16. Levi BP, Yilmaz OH, Duester G, Morrison SJ. Aldehyde dehydrogenase 1a1 is dispensable for stem cell function in the mouse hematopoietic and nervous systems. Blood. 2009; 113:1670–1680. doi:blood-2008-05-156752 [pii]10.1182/blood-2008-05-156752. [PubMed: 18971422]
17. Rovira M, et al. Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. Proc Natl Acad Sci U S A. 2010; 107:75–80. doi:0912589107 [pii]10.1073/pnas.0912589107. [PubMed: 20018761]
18. Tiscornia G, Vivas EL, Belmonte JCI. Diseases in a dish: modeling human genetic disorders using induced pluripotent cells. Nat Med. 2011; 17:1570–1576. [PubMed: 22146428]
19. Sison-Young RLC, et al. Human Pluripotent Stem Cells for Modeling Toxicity. Adv Pharmacol. 2012; 63:207–256. [PubMed: 22776643]
20. Ebert AD, Liang P, Wu JC. Induced Pluripotent Stem Cells as a Disease Modeling and Drug Screening Platform. J Cardiovasc Pharm. 2012; 60:408–416.
21. Inoue H, Yamanaka S. The Use of Induced Pluripotent Stem Cells in Drug Development. Clin Pharmacol Ther. 2011; 89:655–661. [PubMed: 21430656]
22. Cherry ABC, Daley GQ. Reprogrammed Cells for Disease Modeling and Regenerative Medicine. Annu Rev Med. 2013; 64:277–290. [PubMed: 23327523]
23. Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells. 2002; 20:11–20. [PubMed: 11796918]
24. Shapiro, HM. Practical flow cytometry. 4th edn.. Wiley-Liss; 2003.
25. Vaidyanathan G, et al. Targeting aldehyde dehydrogenase: a potential approach for cell labeling. Nucl Med Biol. 2009; 36:919–929. [PubMed: 19875048]
26. Hess DA, et al. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. Blood. 2004; 104:1648–1655. [PubMed: 15178579]
27. Garaycoechea JI, et al. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature. 2012; 489:571–575. doi:10.1038/nature11368nature11368 [pii]. [PubMed: 22922648]
28. Hassner A, Birnbaum D, Loew LM. Charge-Shift Probes of Membrane-Potential - Synthesis. J Org Chem. 1984; 49:2546–2551.
29. Rovira M, et al. Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. P Natl Acad Sci USA. 2010; 107:75–80.

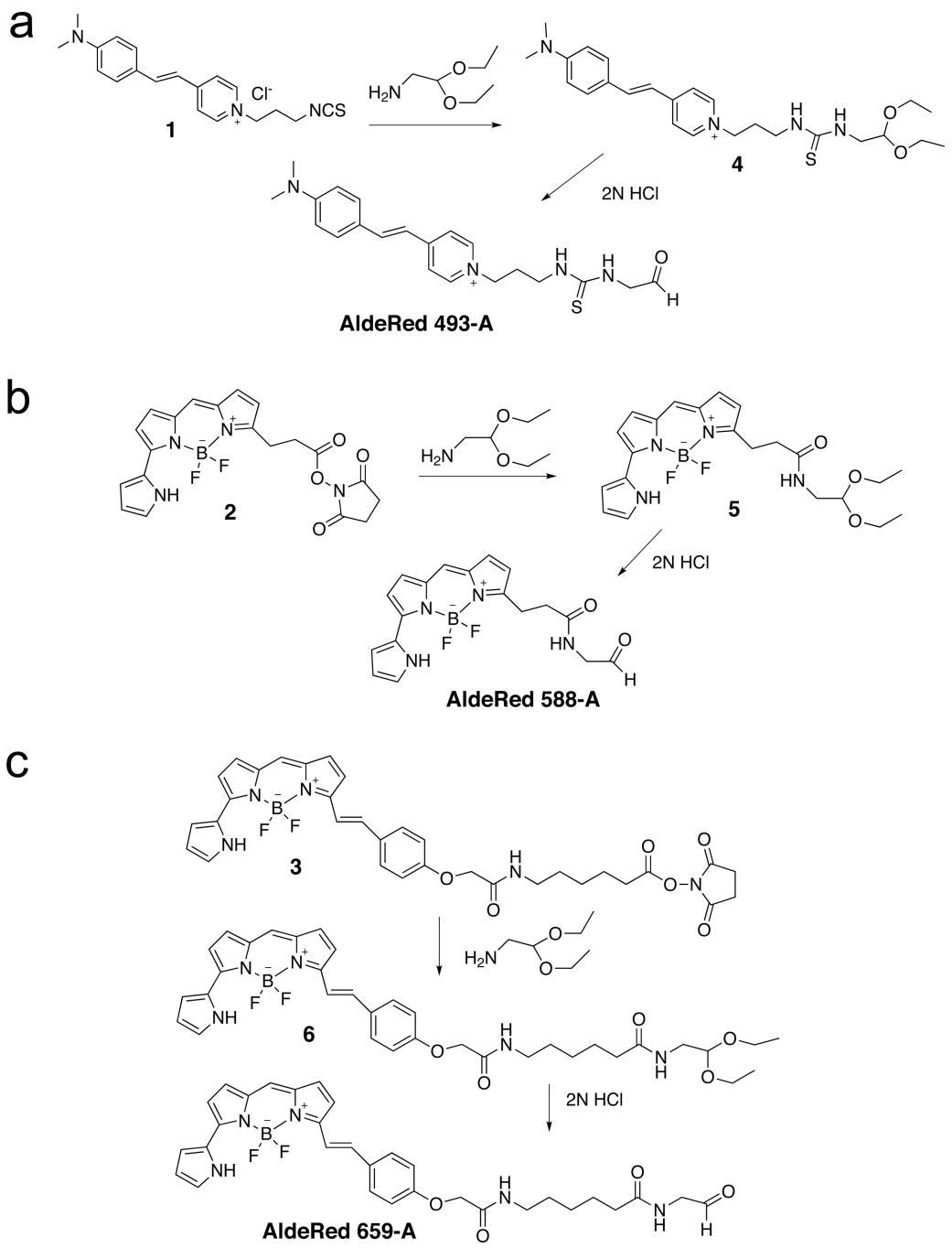

Figure 1.

Synthesis of candidate red-shifted fluorescent substrates for aldehyde dehydrogenase (ALDH). (**a**) AldeRed 493-A. (**b**) AldeRed 588-A, (**c**) AldeRed 659-A. Each compound was prepared as the diacetal (**4**, **5**, and **6**) and deprotected to the aldehyde prior to use.

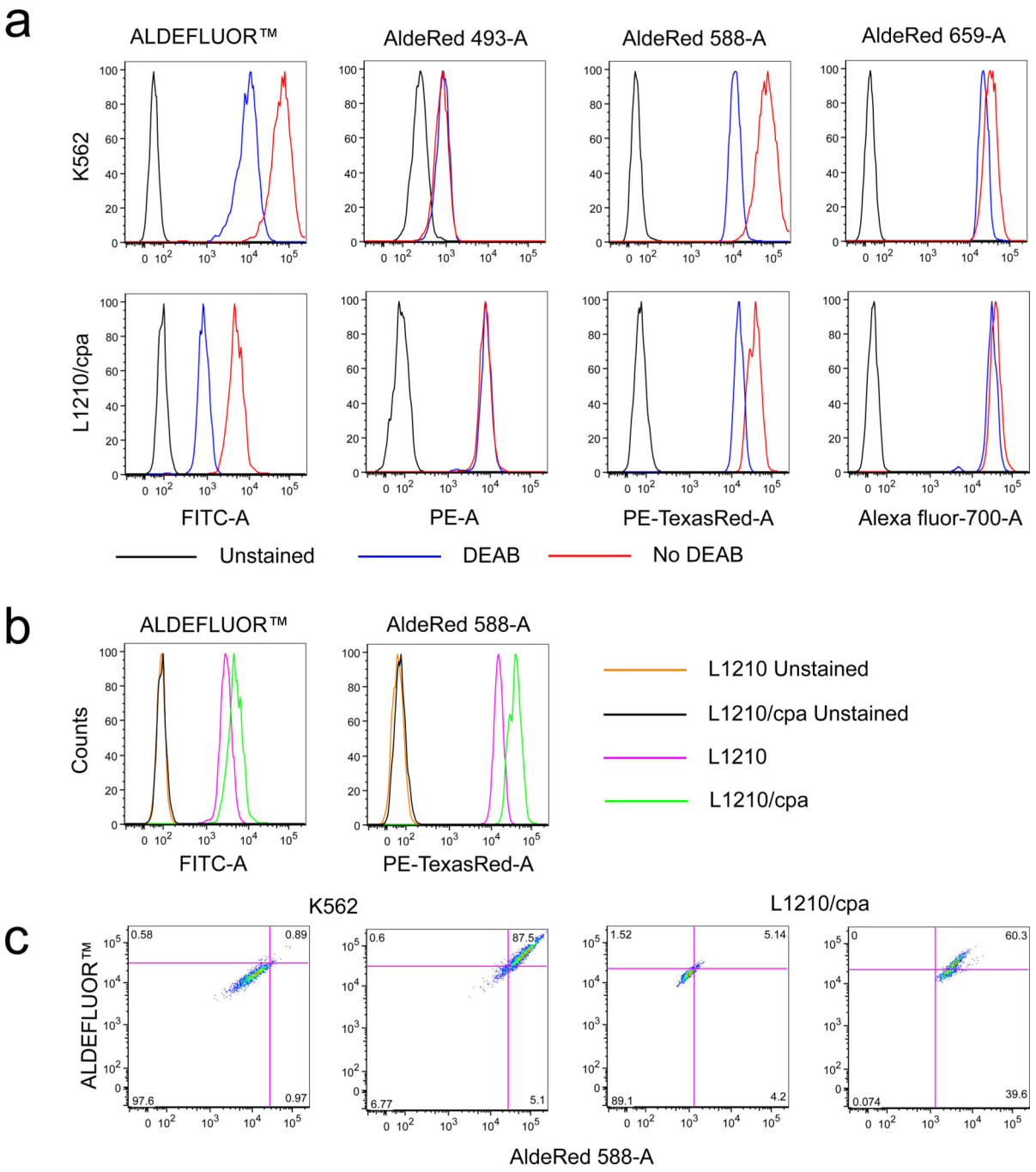

Figure 2.

AldeRed 588-A is a specific substrate for ALDH. (**a**) Fluorescent candidates and the ALDEFLUOR™ reagent were tested with K562 and L1210/cpa cells. The x-axis represents selected detection filters of the LSRII FACS system. (**b**) AldeRed 588-A and the ALDEFLUOR™ reagent tested with L1210/cpa and L1210 cells. (**c**) Co-staining of AldeRed 588-A and the ALDEFLUOR™ reagent with K562 and L1210/cpa cells. DEAB: diethylaminobenzaldehyde.

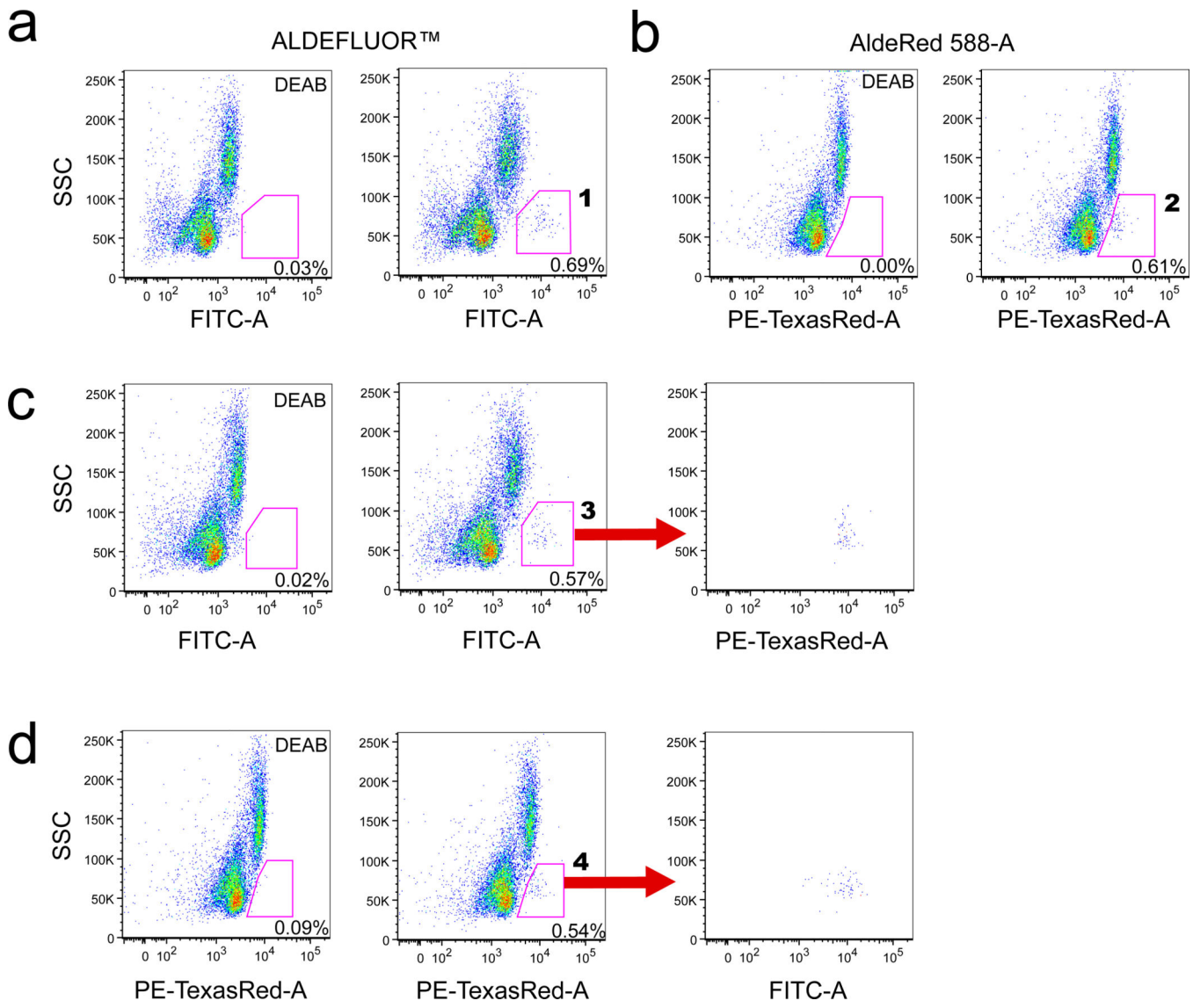

Figure 3.

AldeRed 588-A successfully enables isolation of ALDH^{hi} hematopoietic stem cells (HSCs) from human cord blood mononuclear cells. (**a**) ALDEFLUOR™, (**b**) AldeRed 588-A, (**c** and **d**) double staining with ALDEFLUOR™ and AldeRed 588-A. Red boxes represent gating for sorting ALDH^{hi} cells (**1** and **2**). Numbers at the bottom represent percentage of gated cells. ALDH^{hi} cells (**3** and **4**) were present in the other channels.

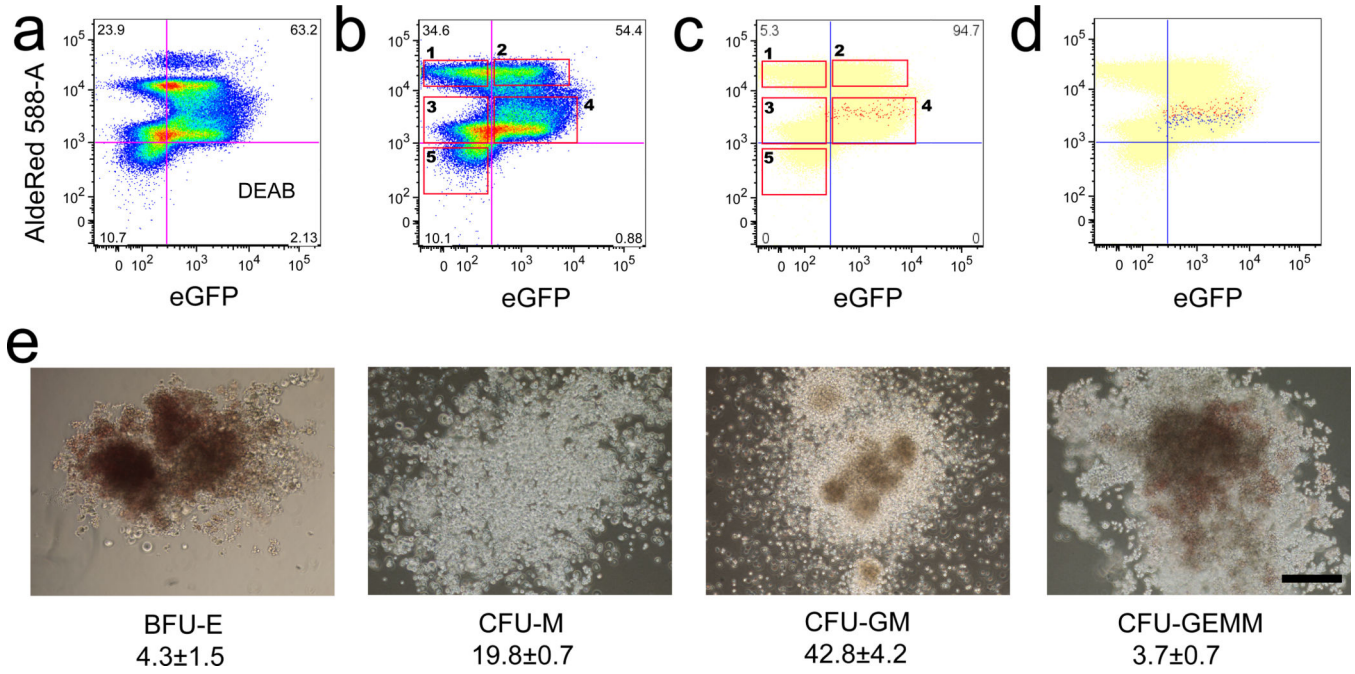

Figure 4.

AldeRed 588-A can enable fractionation of ALDH^{pos} cells from bone marrow (BM) cells from mice that express eGFP. (**a**–**d**) Fractionation of BM cells based on eGFP expression and AldeRed 588-A uptake levels (numbers represent percentage of each quadrant fraction). (**a**) DEAB control. (**b**) Sorting of BM cells into five populations (red box) for colony-forming assays of sorted cells. (**c** and **d**) Enrichment of lineage^{-} /c-Kit^{+}/Sca1^{+} (LKS) hematopoietic stem/progenitor cells (HSPCs) within BM cells. LKS cell populations [red dots for untreated (**c**) and blue dots for DEAB-treated cells (**d**)] were back gated on total BM cells (yellow). The gating strategy (red boxes) for the colony forming assay in (**b**) is superimposed on LKS and BM cell populations (**c**). (**e**) Representative photos of colony-forming cell assays. BFU-E: burst forming unit-erythroid; CFU-GEMM: colony forming unitgranulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM: colony forming unitgranulocyte, monocyte; CFU-M: colony forming unit-megakaryocyte. Numbers represent colonies formed per 20,000 eGFP^{pos}/ALDH^{int} cells (**4**) ± standard deviation, n=6. Size bar: 100 μm.

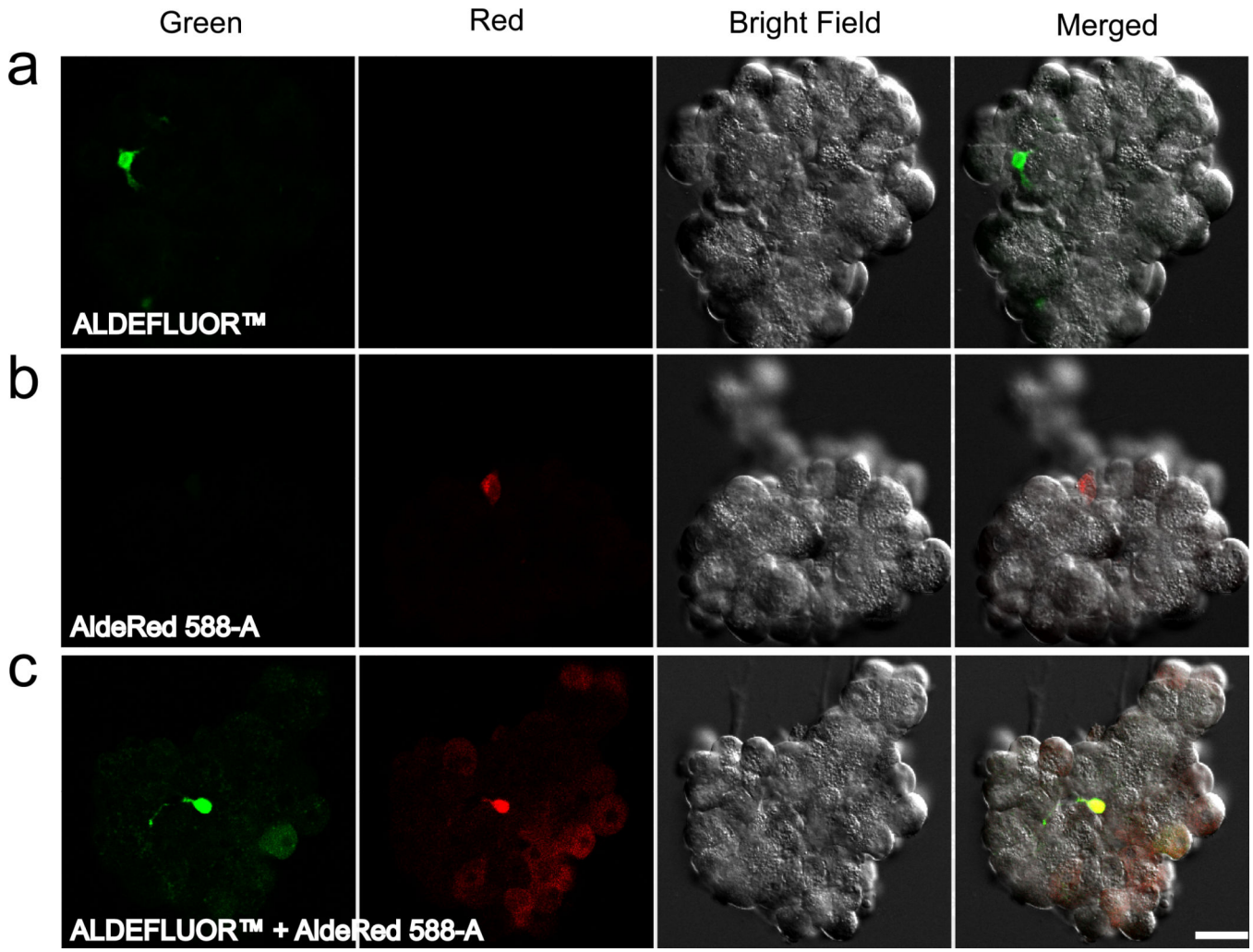

Figure 5.

AldeRed 588-A stains ALDH^{hi} murine pancreatic centroacinar and terminal duct (CA/TD) cells. Confocal microscopic images of murine pancreatic CA/TD cells stained with the ALDEFLUOR™ reagent (**a**), AldeRed 588-A (**b**), and co-stained with both reagents (**c**). Size bar: 20 μm.

Table 1

Colony-forming cell assays with isolated ALDH^{hi} cells (**1** and **2**).

Type	1	2
BFU-E	38.7±5.9	39.5±3.2
CFU-G	21.5±2.6	23.7±4.2
CFU-M	16.2±2.5	15.8±1.8
CFU-GM	10.3±1.6	10.8±2.3
CFU-GEMM	5.3±1.6	5.7±1.4

Numbers represent colonies formed per 500 ALDH^{hi} cells ± standard deviation, n=6.

Note: Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications.