

Flow cytometric screening of aldolase catalytic antibodies

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Abstract—High-throughput screening of cells expressing active catalytic antibody clones by flow cytometry is described. A fluorogenic retro-aldol retro-Michael substrate was designed and synthesized with incorporation of a chloromethyl moiety for intracellular retention. Hybridoma or transfected mammalian cells expressing catalytic antibody molecules could be rapidly isolated.
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Engineering of protein catalysts such as natural enzymes and catalytic antibodies can yield proteins with novel or improved functions that are of significant practical and scientific interest. While rational design has been successful for the engineering of many proteins, the desired change of protein function or property is often elusive even with the structural and biochemical knowledge of the target protein. Moreover, there are a great number of proteins whose structure or mechanism of action is not fully known or understood. Improvement or modification of functions can also be achieved without detailed knowledge about the target protein by directed evolution. Random libraries, constructed by techniques such as error-prone PCR or DNA shuffling, are subjected to genetic selection or high-throughput screening to identify and isolate mutant clones with desired properties. While directed evolution is a powerful technique, lack of a sensitive and efficient screening method often limits its applicability.

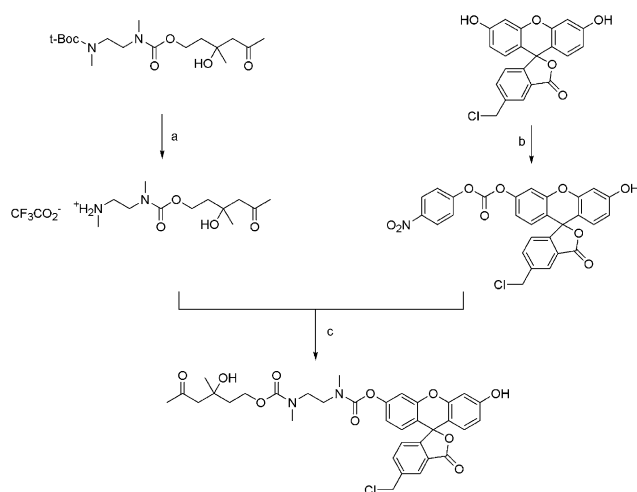
One of the more recent advancements in library screening technology is the use of flow cytometry, which allows for the highly sensitive and efficient detection of protein functions at the level of single cells.

Fluorescence-activated cell sorting (FACS) has been used for screening of antibody^{1,2} and enzyme^{3–7} libraries. FACS is a powerful method for the selection of new catalysts from protein libraries, provided that a sufficiently sensitive fluorogenic substrate is available. Herein we report the development of a new flow cytometry-based method for the selection of catalytic antibodies. Aldolase antibodies previously generated in our laboratory were chosen to demonstrate the efficacy of this method. The aldolase antibodies are highly efficient catalysts that accept a wide variety of substrates, making them suitable for this study.⁸ Our laboratory continues to search for better aldol catalysts and a high-throughput process will be very useful for the routine screening of new antibodies.

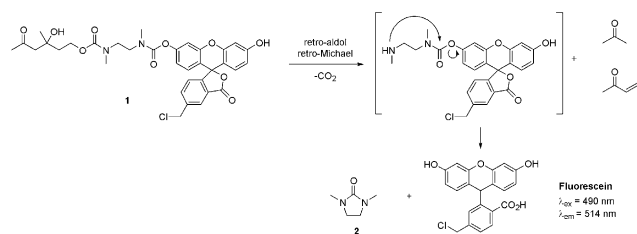
The screening of a catalytic antibody library by the flow cytometry assay requires a fluorogenic substrate, which upon catalytic reaction will generate a fluorescent compound. It is desirable that the new FACS substrates have the following properties: a low cellular background; a fluorophore with a high quantum yield, preferably excited at 488 nm (the major excitation wavelength used in flow cytometers); a relatively long linker, which extends the reaction site away from the bulky fluorophore; and cellular retention of the product fluorophore. With these requirements in mind, the fluorescein derivative **1** was synthesized (Scheme 1).⁹ Upon reaction with antibody Ab 38C2, **1** undergoes antibody-catalyzed, tandem retro-aldol retro-Michael reactions, followed by cyclization of the self-immolative linker, releasing urea **2** and 5-chloromethylfluorescein

Keywords: Aldolase; Catalytic antibody; Aldol; Fluorescence-based screening.

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Scheme 1. Synthesis of substrate **1**. Reaction conditions: (a) TFA, 10 min at rt, acid removed in vacuo and immediately added to the reaction mixture *b*; (b) THF, 1 equiv 4-nitrophenylchloroformate, 0 °C, 1 equiv TEA added dropwise, 2 h; (c) excess TEA, rt, 16 h. Product purified by preparative TLC (ethyl acetate). The preparation of the starting materials followed the procedures described in Refs. 15 and 16.



Scheme 2. Retro-aldol, retro-Michael reaction of substrate **1** catalyzed by aldolase antibody 38C2.

(Scheme 2). The tandem reactions significantly reduce the background reaction rate. The chloromethyl moiety reacts with nucleophiles inside the cell (e.g., glutathione or other intracellular sulfhydryl groups), thus retaining the product within the cell.^{6,10,11} Compound **1** was first tested with purified Ab 38C2 and shown to be a substrate for this catalyst, as monitored by the release of 5-chlorofluorescein ($k_{\text{cat}} = 0.018 \text{ min}^{-1}$, $K_m = 22 \mu\text{M}$). Cell-based studies were then undertaken to confirm the specificity of these compounds as probes for aldolase activity. To assess the possibility of sorting cells based on their catalytic activity, hybridoma cells expressing Ab 38C2 or non-catalytic Ab 29B4 were mixed prior to incubation with the substrate **1**. After reacting for 4 h, the cells were analyzed by flow cytometry, as shown in Figure 1.¹² Two distinct populations of cells, with different fluorescence intensities, are clearly observed in this experiment. In addition, the peak ratios correlate to the ratios of catalytic versus non-catalytic cells. These results clearly demonstrate that the discrimination of cells expressing catalytic antibody from cells without catalyst is possible using fluorogenic aldol sensors. The assay was then tested by sorting 10 million cells containing a predetermined mixture of 90% 29B4 and 10%

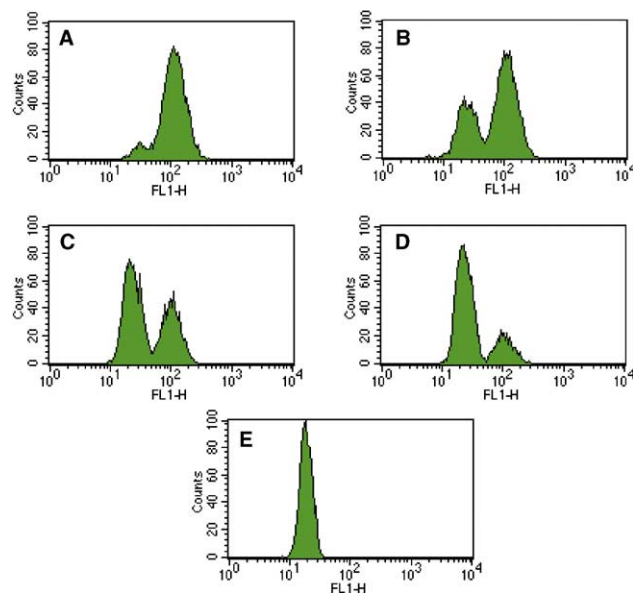


Figure 1. Mixed cultures of JW 38C2 and JW2 29B4 hybridoma cells incubated with $10 \mu\text{M}$ substrate **1** for 4 h. **A**, 38C2 only, **B**, 38C2:29B4 = 3:1, **C**, 38C2:29B4 = 1:1, **D**, 38C2:29B4 = 1:3, **E**, 29B4 only.

38C2-expressing hybridoma cells.¹² After incubation with aldolase substrate **1**, positive cells were recovered by selection of viable cells that exhibited the highest fluorescence intensity at 530 nm (Fig. 2, R3). The 1.1×10^5 positive cells thus obtained were cultured and re-analyzed by flow cytometry to confirm the enrichment of catalytically active cells, as shown in Figure 2 (red histogram).

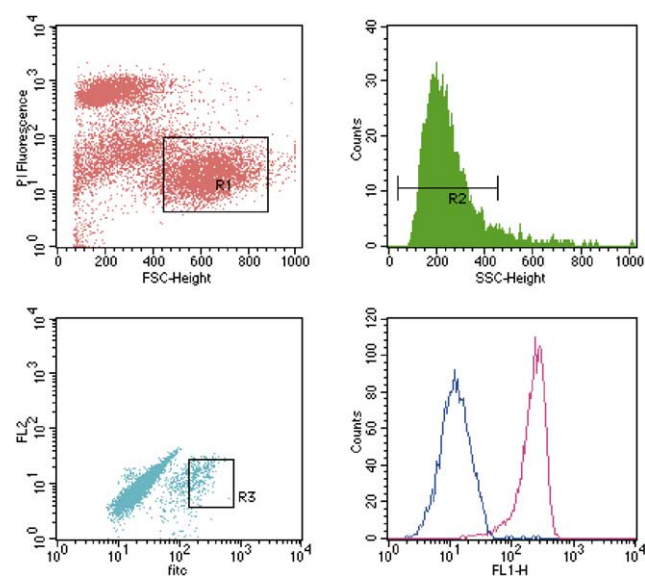


Figure 2. Cells were sorted based on propidium iodide stain (R1), on low-to-moderate side scatter (R2), and on high FL2 signal (R3) were sorted by FACStar Plus. FACS analysis after regrowth of the sorted cells shows high FL2 signal (red histogram) relative to negative control (blue histogram).

The selection of hybridoma cells producing catalytic antibodies is only one possible application for the method described above. Recently developed techniques for the generation of genetic diversity, such as DNA shuffling and mutagenic PCR, have been successfully used to produce libraries from which enzymes with new properties can be evolved. With correct design of fluorogenic substrates a variety of different reactions can be monitored using a linker that separates the site of the reaction from the fluorophore.

We have tested the potential of the method for the isolation of catalysts from a plasmid-encoded library introduced and expressed in mammalian cells. The human 293T cell line was transfected with recombinant 38C2 and analyzed by flow cytometry.^{13,14} As a control, cells were transfected with an inactive mutant of 38C2 cloned into the same expression vector. After incubation with substrate **1** the cellular fluorescence was analyzed by flow cytometry to confirm that the catalytic activity of Ab 38C2 could be detected in the transiently transfected cells and the product fluorescence was well resolved from that of the background reaction in the control cells (Fig. 3).

In conclusion, a novel cell-based assay for the reaction-based selection of aldolase antibodies is presented. Fluorogenic aldol sensors for FACS were designed and synthesized. The reaction sensors could be selectively converted to products that are trapped inside cells expressing functional catalysts, allowing for cell selection by flow cytometry. By using this method it is possible to rapidly screen hybridoma cells in order to identify clones that express catalytic antibodies. In addition, we have also shown that mammalian cells that transiently express recombinant aldolase antibody catalysts can also be selected suggesting that the methodology will be applicable to recombinant library screening as well. Thus this selection should find utility in the identification and evolution of novel aldolase antibodies or enzymes. Since the aldol sensors share the retro-aldol retro-Michael trigger mechanism common to our aldolase activated prodrugs, this methodology may facilitate the selection of catalysts that are optimized with respect to their ability to activate the multireaction

trigger and thus improve the potential of aldolase antibody prodrug therapy.^{15,17}

Acknowledgements

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- JW 38C2 and JW 29B4 cells were counted with a hemocytometer and mixed. 5×10^5 (for analysis) or 1×10^8 (for sorting) mixed cells were incubated in RPMI (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies), antibiotic-antimycotic reagent (Life Technologies) and the substrate **1** (10 μ M from 10 mM DMSO stock) for 4 h at 37 °C in a humidifying atmosphere of 5% CO₂. After incubation the cells were washed once with FACS sorting buffer (1 $\times$ PBS, 1 mM EDTA, 25 mM HEPES, pH 7.0, and 1% dialyzed FBS) and trypsinized and resuspended in 3 mL FACS sorting buffer with 1 μ g mL⁻¹ PI. Cells were analyzed on a FACS can flow cytometer or sorted on a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA). The trigger parameter was set to FSC. The product of substrate **1** was monitored by excitation with an argon ion laser (wavelength, 488 nm) and the emission was detected in the FL-1 channel via a 530/30 nm filter. PI staining of dead cells was monitored by excitation with the same laser and emission detection in the FL-3 channel via a 650 nm long pass filter (FACScan) or 630/22 nm filter (FACStar plus), and the autofluorescence of the cells was detected in the FL-2 channel via a 585/42 nm filter. The cells were gated in FSC versus PI, in FL-1 versus FL-2 and in SSC (Fig. 2). 1.1×10^5 cells were collected in Normal-R mode using a

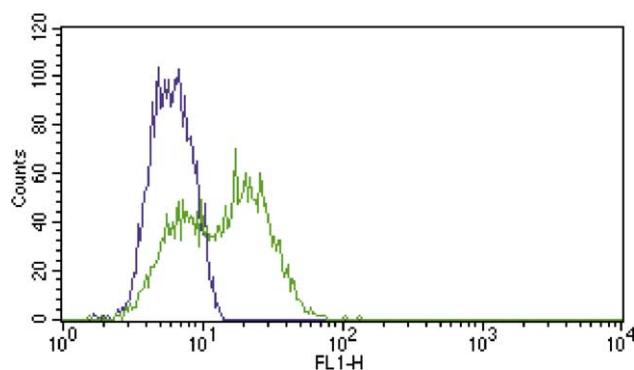


Figure 3. FACS analysis of transiently transfected 293T cells. Green: transfected with 38C2 IgG, blue: negative control.

100 μ m nozzle into 1 mL fetal bovine serum (Life Technologies, Gaithersburg, MD) and recultured for analysis.

13. The V_L and V_H chains of the Ab 38C2 gene were cloned into the pIGG-8 vector for the transfection experiments. As a control, the non-catalytic antibody LM609 cloned into the pIGG-8 vector was used. Human embryonic kidney 293T cells were transfected with pIGG-8 constructs by LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD) according to the procedure recommended by the manufacturer. On the day after transfection, the medium was replaced with fresh medium and substrate **1** was added to a final concentration of 10 μ M. The cells were incubated for additional 8 h then were analyzed or sorted as described above. Positive cells (99 percentile or higher fluorescence) were collected and the antibody genes were obtained in one of the three ways: (1) plasmid was recovered from the cells,¹² (2) antibody gene was amplified by PCR from the isolated plasmid and cloned into pIGG-8 vector, or (3) total RNA was retrieved using TRI reagent (Molecular Research Center) and the antibody gene was amplified by RT-PCR, followed by cloning into pIGG-8 vector. The resulting plasmids were amplified by transformation into XL1-Blue (Stratagene, La Jolla, CA) or SURE (Stratagene, La Jolla, CA) *E. coli* cells and were analyzed by DNA sequencing, analytical PCR or FACS analysis.
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