

Enrichment of Cell-Targeting and Population-Specific Aptamers by Fluorescence-Activated Cell Sorting**

Marie-Sophie L. Raddatz, Andreas Dolf, Elmar Endl, Percy Knolle, Michael Famulok,* and Günter Mayer*

Dedicated to Professor Gernot Boche on the occasion of his 70th birthday

The reliable targeting of distinct cells and defined subpopulations of cells is an important task in chemical biology and medicine. Therefore, sophisticated reagents are required that facilitate the specific and high-affinity binding of a defined cell and, more importantly, the identification of a cell subpopulation with particular features, for example, malignant transformation. Besides monoclonal antibodies, which detect certain cell-surface-associated molecules specifically, nucleic acid aptamers are a class of molecules that fulfill the criteria for the specific targeting of cells. Aptamers are short single-stranded nucleic acids that bind with high affinity and high specificity to a target molecule of choice. They can be isolated by *in vitro* selection, or SELEX (systematic evolution of ligands by exponential enrichment).^[1]

Aptamers can target peptides, proteins, and RNA, but also heterogeneous targets,^[1d,2] such as viruses, parasites, bacteria, and immortalized tumor cells. One aim of researchers is to generate aptamer-based molecular probes.^[2b,3] The most critical step within the *in vitro* selection process is the separation of target-bound from nonbound or nonspecifically bound nucleic acid species. Therefore, specific separation techniques which do not disrupt the target–nucleic acid complex nor favor the isolation of nonspecific nucleic acids are implemented in the selection protocol. Sophisticated procedures for the immobilization of homogenous molecules enable the efficient separation of bound from nonbound nucleic acids. Similarly, adhesive cells can be addressed by simply washing them after incubation with the nucleic acid

library.^[4] In contrast, cells growing in suspension were subjected to *in vitro* selection by centrifugation to separate bound from nonbound aptamers.^[5] This procedure, however, has two distinct disadvantages: 1) Centrifugation damages cells, and 2) this approach does not discriminate between dead cells with reduced cell-membrane integrity and those which are vital. Furthermore, dead cells reveal a sequence-independent strong affinity for nucleic acids (see Figure 1 c,d in the Supporting Information). This property leads to inefficient progress of the selection experiment: A large number of selection cycles (> 20) are required to enrich an aptamer population.^[5] Increasing numbers of selection cycles favor, in turn, the enrichment of unwanted sequences. These sequences are superior replicators because they adapt to the enzymatic amplification steps of the selection process rather than to target binding.^[6] Therefore, a dramatic shift occurs within the selected nucleic acid population towards non-specific nucleic acids, which often outcompete specific aptamers to result in the failure of the SELEX experiment. Thus, although successful in individual cases, these selection schemes are far from being generally applicable. An efficient selection process that avoids these disadvantages requires the directed targeting of an appropriate subpopulation of cells.

To address this issue, we report herein a novel and sophisticated approach in which fluorescence-activated cell sorting (FACS) is implemented in the selection procedure. FACS can be used to separate distinct subpopulations of cells from other subpopulations within composite cell mixtures. Vital and dead cells, for example, can be discriminated and separated on the basis of their different light-scattering characteristics or their ability to convert cell-permeable dyes into a fluorescent form enzymatically.^[7] We hypothesized that a combination of the FACS technology with *in vitro* selection might enable the direct targeting of a certain subpopulation of cells, such as dead or vital cells, within a cultured cell mixture.

We first implemented a live-cell/dead-cell separation step in the selection process by using a digital high-speed cell sorter. We performed an *in vitro* selection experiment that targeted vital Burkitt lymphoma B cells, whereby FACS was used to separate bound from nonbound nucleic acids (Figure 1). This FACS–SELEX procedure is guided simply by the association of members of the nucleic acid library with a vital cellular phenotype, rather than, as in the case of most previously reported selection protocols, by the high affinity of nucleic acids for composite cell mixtures. Ten rounds of *in vitro* selection were carried out with this novel FACS–SELEX

[*] M.-S. L. Raddatz, Prof. Dr. M. Famulok, Dr. G. Mayer
LIMES—Life and Medical Science, Universität Bonn
Gerhard-Domagk-Strasse 1
53121 Bonn (Germany)
Fax: (+49) 228-73-4809
E-mail: m.famulok@uni-bonn.de
gmayer@uni-bonn.de

A. Dolf, Dr. E. Endl, Prof. Dr. P. Knolle
Institute of Molecular Medicine and Experimental Immunology
Universität Bonn
Sigmund-Freud-Strasse 25, 53105 Bonn (Germany)

[**] This research was supported by research grants from the DFG Sonderforschungsbereich 704 (to M.F.) and the Fonds der Chemischen Industrie (to M.F. and G.M.) We thank Nicole Kuhn for excellent technical assistance and Jens Müller for support during the preparation of peripheral blood mononuclear cells from whole blood.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.200800216>.

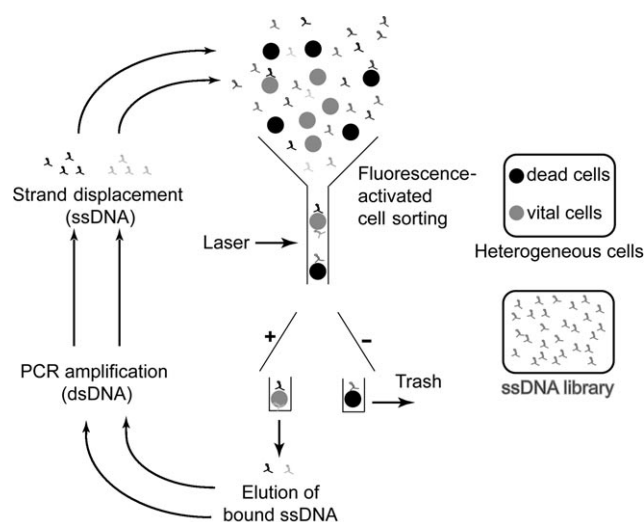


Figure 1. FACS-SELEX scheme. Fluorescence-activated cell sorting (FACS) is used to separate bound from nonbound nucleic acids.

Composite cell mixtures, which comprise dead cells with reduced membrane integrity and vital cells, are incubated with an ssDNA library. FACS sorts the dead cells from the vital cells and thereby selects bound nucleic acids associated with the vital-cell phenotype. The selected nucleic acids can be amplified by PCR. After single-strand displacement, the resulting ssDNA library serves as a starting library for the next selection cycle. ss = single-stranded, ds = double-stranded.

scheme. An enriched nucleic acid library resulted that revealed enhanced binding to vital Burkitt lymphoma cells relative to that of the starting library (Figure 2a).

As expected, cloning and sequencing of the enriched nucleic acid library revealed several monoclonal DNA sequences, which can be grouped into distinct families. One representative clone, C10, was analyzed further. By using fluorescence-labeled C10, we determined a dissociation constant of 49.6 ± 5.5 nM for the interaction between C10 and Burkitt lymphoma cells, whereas a scrambled control sequence (C10sc) labeled in the same way showed almost no binding (Figure 2b,c). The same behavior was observed for C10 and C10sc in an assay format based on radioactively labeled nucleic acids (see Figure 3 in the Supporting Information). Remarkably, no interaction with an acute-T-cell-leukemia cell line was detected (see Figure 3 in the Supporting Information). Furthermore, C10 binds spe-

cifically to vital CD19⁺ Burkitt lymphoma cells (CD19 is a B-cell-specific cell-surface-marker protein), whereas the control DNA sequences C11 and C10sc show almost no binding (Figure 3a; see also Figure 4a in the Supporting Information).

To test whether C10 can discriminate between these lymphoma cells and nonmalignant CD19⁺ B cells, we first defined the lymphocyte subpopulation within a population of peripheral blood mononuclear cells (PBMCs) from a healthy volunteer on the basis of forward-/side-scatter analysis (Figure 3b, gray ellipse, and Figure 4b in the Supporting Information, gray ellipse). From this subpopulation, the vital CD19⁺ B cells were defined by staining with calcein acetoxymethyl ester (calcein AM), a fluorophore that is activated by cells that undergo metabolism, and anti-CD19 antibodies labeled with allophycocyanin (APC; Figure 3c, gray circle, and Figure 4c in the Supporting Information, gray circle). Relative to the control DNA molecules C11 and C10sc, C10 shows no enhanced binding to this nonmalignant CD19⁺

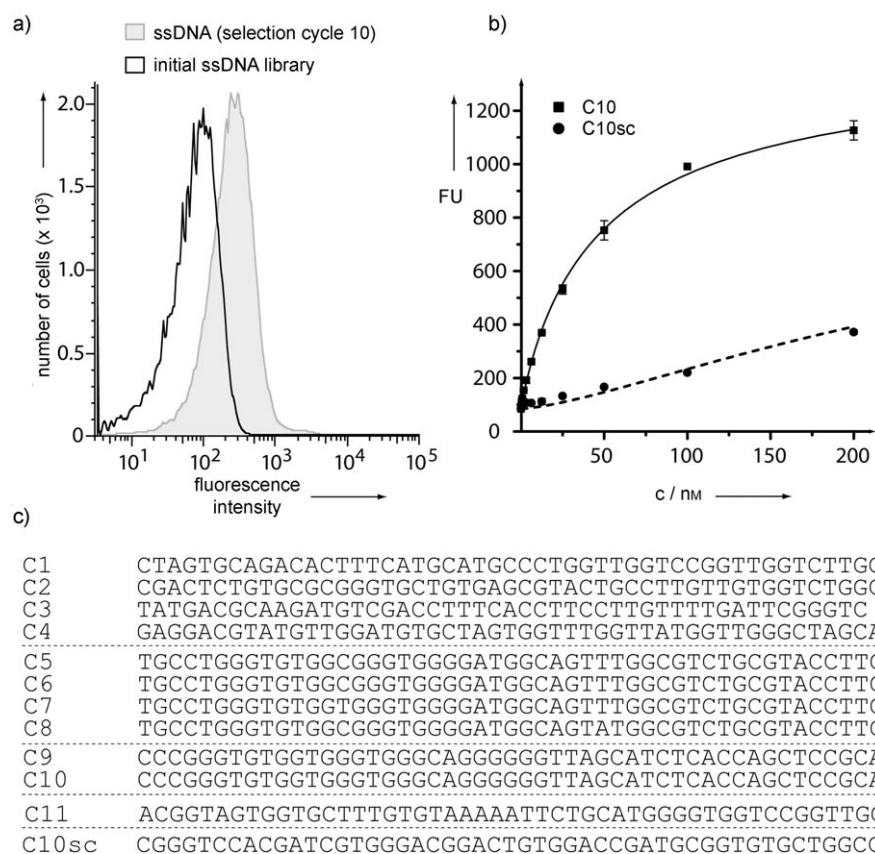


Figure 2. Enrichment of an ssDNA population that binds vital Burkitt lymphoma cells. The histograms show the fluorescence-intensity distribution of cells incubated with fluorescein-conjugated ssDNA libraries. a) Overlaying of the flow cytometric data demonstrates that the selected ssDNA library from cycle 10 (gray) shows enhanced binding to vital Burkitt lymphoma cells relative to that of the starting ssDNA library (black line; x coordinate: fluorescence intensity of bound DNA). b) Determination of the dissociation constant of fluorescence-labeled C10 and the scrambled variant C10sc. FU: Mean fluorescence. c) Representative sequences of the selected ssDNA library obtained by FACS-SELEX targeting of vital Burkitt lymphoma cells. Only the sequences of the initial random regions are shown. C11: nonbinding ssDNA negative control sequence. C10sc: nonbinding ssDNA with an identical nucleotide composition to that of C10 but a different primary sequence.

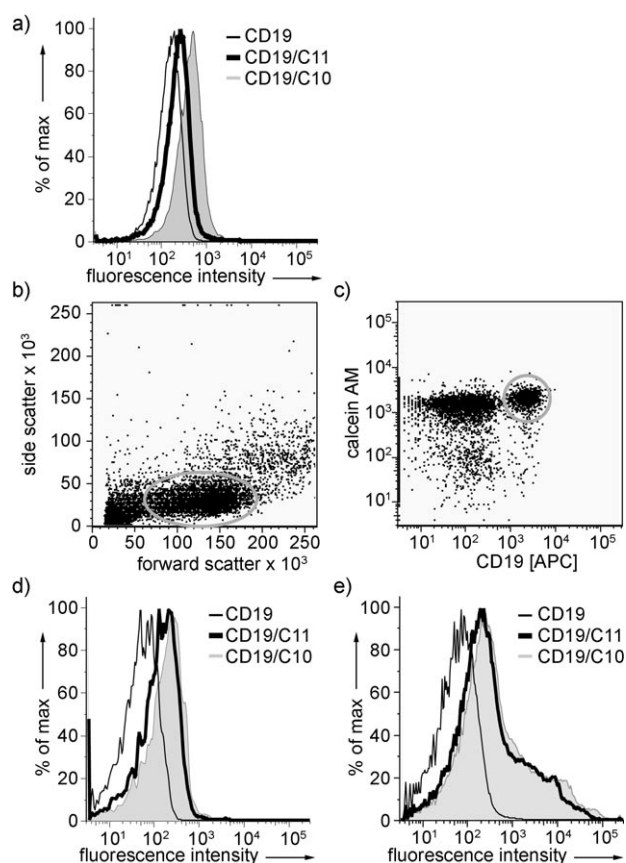


Figure 3. Aptamer C10 binds specifically to vital Burkitt lymphoma cells. a) The affinity of fluorescein-conjugated ssDNA was analyzed by flow cytometry, which revealed specific binding of C10 to vital CD19⁺ Burkitt lymphoma cells (gray integral); C11 (thick black line) and unstained cells (thin black line) showed almost no binding (y coordinate: % of max is the normalized percentage of cells that show a certain fluorescence intensity due to autofluorescence and bound fluorescence-labeled DNA). Vital Burkitt lymphoma cells were gated with an APC-labeled anti-CD19 antibody. b) B cells within the heterogeneous PBMC population were first identified by setting a gate on the lymphocyte population within the forward scatter versus side scatter (gray ellipse). c) Vital lymphocytes (that is, the cells within the ellipse in (b)) were defined more precisely (gray circle) by an additional analysis gate in a two-parameter display with anti-CD19 [APC] versus calcein AM. d) The overlaying of fluorescence-intensity histograms reveals that C10 (gray line) and C11 (thick black line) bind similarly to the vital B-cell population defined in (b) and (c), and with somewhat higher affinity than the unstained control cells (thin black line). e) Histograms of ungated cells (the entire CD19⁺ cell population, including dead cells).

B-cell subpopulation (Figure 3d, and Figure 4d in the Supporting Information). For comparison, we also examined the population of CD19⁺ cells from PBMCs that included dead cells, and found that dead primary cells show nonspecific binding to C10, C11, and C10sc, as indicated by the broad shoulder above the fluorescence intensity of 10^3 (Figure 3e, and Figure 4e in the Supporting Information). This observation is in accordance with the data obtained with dead Burkitt lymphoma cells (Figure 1d,f in the Supporting Information) and further underlines the importance of actively excluding

dead cells both in the SELEX experiment and in subsequent analysis of aptamers obtained.

Taken together, these results demonstrate that the aptamer C10 is a B-cell-tumor-targeting DNA aptamer that distinguishes between Burkitt lymphoma B cells and primary B cells. However, the target molecule of the aptamer C10 and the potential of C10 as a tumor-specific agent remain to be determined.

We next compared the flow cytometry selection scheme with a selection protocol that utilizes centrifugation as the separating step. The latter selection resulted in a nucleic acid population that merely reflects the nonspecific dead-cell-binding behavior of the starting library (see Figure 1c,d in the Supporting Information). Even after 20 rounds of in vitro selection, no enhanced binding of the selected library B20 to vital Burkitt lymphoma cells was detected (see Figure 1e,f in the Supporting Information). The low selection pressure during the selection process against composite cells comprising both vital and dead cells results in an inefficient separation of bound from nonbound nucleic acid species. Thus, this separation step is incapable of isolating only the vital cell-binding high-affinity aptamers, which are rare relative to the sequences that bind nonspecifically. Instead, nonspecific nucleic acids will be coselected and will eventually overwhelm the specific nucleic acids, thus hampering the enrichment of aptamers for vital cells. We applied the DNA library B20 to further selection cycles with the FACS SELEX scheme. After six further selection cycles, the resultant DNA library, termed DNA No. 45, was shown to bind to vital Burkitt lymphoma cells (see Figure 2 in the Supporting Information). These results provide further evidence for the suitability of the FACS-SELEX approach for the efficient targeting of a subpopulation of cells and the streamlining of in vitro selection against complex targets.

In conclusion, we have shown that FACS-SELEX serves as an efficient, rapid, and robust route for the selection of cell-specific aptamers. Our new in vitro selection method requires only a low number of selection cycles and is therefore less susceptible to selection artifacts than conventional whole-cell SELEX. FACS-SELEX introduces a new dimension to the isolation of functional nucleic acids: It relies essentially on the association of a certain nucleic acid sequence with a defined phenotype or subpopulation of cells, rather than solely binding to a given target with high affinity. To our knowledge, we have described the first example of the implementation of flow cytometry and cell sorting in the selection of nucleic acid aptamers. This selection process will be accessible to clinical laboratories for the assessment of high-affinity and specific cell-targeting agents, which are complementary to widely used antibodies. More importantly, it paves the way for the selection of tumor- and cell-specific aptamers that target distinct subpopulations of cells in heterogeneous composite mixtures of cells isolated directly from primary tissues or body fluids. This method may be a first step towards the use of aptamers for individualized diagnostic and medical applications.

Experimental Section

FACS–SELEX: In the first selection cycle, the synthetic ssDNA library D1 (75 pmol) was incubated with 1×10^6 Burkitt lymphoma cells in SELEX buffer (1 mL; $1 \times$ HBSS (Hank's buffered salt solution) supplemented with MgCl_2 (1.4 mM)) at 37°C together with bovine serum albumin ($1 \mu\text{g} \mu\text{L}^{-1}$) and salmon-sperm DNA ($1 \mu\text{g} \mu\text{L}^{-1}$). The desired vital subpopulation of the B cells was then separated according to its properties in flow cytometry by FACS (FACS DiVa, Becton Dickinson, Heidelberg). The isolated ssDNAs were eluted by heat denaturation for 5 min at 95°C , extracted with phenol–chloroform, and subjected to gel filtration (G25). After PCR amplification and strand displacement (see the Supporting Information), the resulting ssDNAs served as the starting library for the next cycle.

Received: January 16, 2008

Published online: June 2, 2008

Keywords: aptamers · cell recognition · flow cytometry · fluorescence · in vitro selection

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