Fluorescence-activated cell sorting for aptamer SELEX with cell mixtures

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Aptamers that target a specific cell subpopulation within composite mixtures represent invaluable tools in biomedical research and in the development of cell-specific therapeutics. Here we describe a detailed protocol for a modular and generally applicable scheme to select aptamers that target the subpopulations of cells in which you are interested. A fluorescence-activated cell-sorting device is used to simultaneously differentiate and separate those subpopulations of cells having bound and unbound aptamers. There are fewer false positives when using this approach in comparison with other cell-selection approaches in which unspecific binding of nucleic acids to cells with reduced membrane integrity or their unselective uptake by dead cells occurs more often. The protocol provides a state-of-the-art approach for identifying aptamers that selectively target virtually any cell type under investigation. As an example, we provide the step-by-step protocol targeting CD19$^+$ Burkitt's lymphoma cells, starting from the pre-SELEX (systematic evolution of ligands by exponential amplification) measurements to establish suitable SELEX conditions and ending at completion of the SELEX procedure, which reveals the enriched single-stranded DNA library.

INTRODUCTION

Targeting distinct cells or cell subpopulations in the complex environment of cell mixtures, tissues or whole organisms constitutes one of the biggest challenges in molecular biomedicine. This requires the development of sophisticated molecular ligands. A widespread class of mol ecules for this purpose is represented by antibodies and their engineered descendants[1–3]. These have proven to be extremely useful and versatile tools for a large variety of applications in biomedicine, biotechnology, diagnostics and treatment; however, chemical synthesis and strategic modification of antibodies is a complicated, challenging and laborious task[4,5]. In contrast, nucleic acid aptamers have been shown to be accessible entirely by chemical synthesis. Furthermore, aptamers can be easily modified with chemical groups[6–8] that increase their stability in various biological compartments that are otherwise harmful for a nucleic acid[9]. Chemical groups can also serve as handles for attaching functionality that reduces clearance, as reporters for imaging or as attachment points for molecular cargo that is transported by each aptamer to its specific site of action. Aptamers thus represent an emerging class of molecules that can be used for many different cell-targeting applications[10–16].

Aptamers are single-stranded synthetic oligo(deoxy)nucleotides[17,18] that adopt distinct three-dimensional shapes similar to proteins and that can be used for molecular recognition[19–22]. Aptamer structures consist of binding pockets and clefts that enable this class of functional nucleic acids to specifically and tightly bind to a variety of diverse molecular targets, ranging from simple ones such as small organic molecules[23–25], ions[26], peptides[27,28], proteins[29], nucleic acids[30–33] and other macromolecules to complex targets such as higher-order protein complexes, whole cells, viruses, parasites or tissues. These features mean that aptamers have the potential to be excellent tools to target pathogenic and malignant cells or tissues.

Aptamers are obtained by a repetitive *in vitro* selection process, also termed SELEX (systematic evolution of ligands by exponential amplification)[17,18]. SELEX comprises four steps: incubation with target molecule, separation of bound from unbound species, elution and, finally, amplification of the bound nucleic acids[34]. The likelihood that aptamers can be generated for a defined target cannot be reliably given. However, according to our experiences (>300 selections against homogenous targets), a conservative estimate of the success rate of *in vitro* selection experiments that target homogenous molecules is ~50%. The selection of aptamers against complex structures is, in turn, more difficult, and there are fewer examples in the literature. Aptamers that target a defined cell population can be obtained using a number of different procedures. First, purified cell-specific proteins known to be selectively present on malignant cells can be used as target molecules for SELEX[35,36]. However, this procedure requires that the target protein is known and can be purified without loss of function. At least in the case of transmembrane receptors, the latter is a laborious task. If the target and its extracellular epitopes are known, a defined epitope can be used in an *in vitro* aptamer selection. This strategy bears the fairly high risk that the aptamer loses its binding activity if the same epitope is presented in the context of the entire, membrane-embedded receptor. Alternatively, a receptor of interest can be expressed recombinantly on cells that naturally do not express it. These cells can then be used as target cells during SELEX, whereby cells that do not express the same target need to be used for preselection procedures to eliminate sequences with unspecific cell-binding behavior[37]. This approach has the disadvantage that the following requirements must be met: (i) the target must be known and cloned, and a stable target-overexpressing cell line must be generated and (ii) an identical cell line that does not (over)express the same target must be available or generated in parallel. Furthermore, owing to the presence of many other potential target molecules on the cell surface, it may result in aptamers that recognize other than the intended recombinant target. A third approach directly employs pathogenic cells as targets during the selection process[38]. This approach has the advantage of targeting cell-surface molecules within their native environment and without requiring prior knowledge of their identity.

The successful isolation of aptamers that specifically target a defined subpopulation of cells by *in vitro* selection of vast

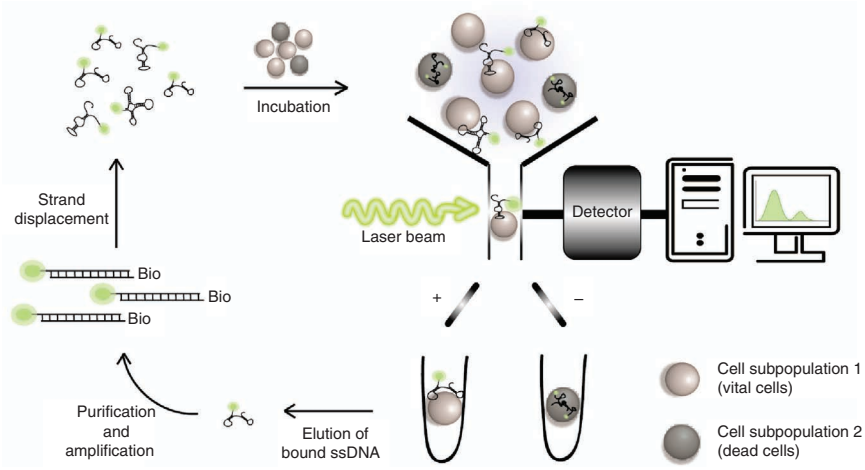

Figure 1 | Selection scheme based on FACS. Composite cell mixtures consisting of both vital and dead cells with reduced membrane integrity are incubated with an ssDNA library. Dead and vital cells can be separated in a cell sorter according to the differences in metabolic activation of calcein AM. This procedure selects aptamers bound to the cell surface that are associated with the vital cell phenotype.

combinatorial oligo(deoxy)nucleotide libraries depends on two prerequisites. First, the cells of interest need to be accessible. Many can be obtained as immortalized cell lines that are maintained by standard cell culturing methods. Otherwise, primary cell isolates from tissues can be prepared and stored until used for selection cycles. Second, cells with reduced membrane integrity, i.e., those undergoing apoptosis or necrosis, need to be eliminated. These cells take up nucleic acids unselectively, thereby counteracting the success of the SELEX process (**Fig. 1**).

Sefah *et al.*[39] recently published a cell-SELEX protocol that reported the separation of bound from unbound single-stranded DNA (ssDNA) molecules using mainly centrifugation during the selection process. In case of homogeneous cell populations that grow in solution, this protocol may represent a suitable route to cell-specific aptamers. An advantage of this approach is that it does not require the application of cost-intensive equipment (such as a cell sorter). On the contrary, a limitation of separating functional from nonfunctional aptamer sequences by centrifugation is that cells with reduced membrane integrity may not be efficiently excluded. This potentially counteracts the enrichment of cell-specific aptamer sequences, because aptamer sequences that have randomly entered these cells may not be removed. Indeed, the large number of selection cycles required to enrich aptamers by centrifugation (>20) might argue for this possibility. We initially employed an approach similar to the one reported by Sefah *et al.*, to target Burkitt's lymphoma cells, but even after 20 selection cycles, no enrichment of cell-specific aptamers could be observed[40]. After thorough analysis, we revealed that the dead cells present within our cell culture preparations show a high tendency to unselectively carry nucleic acids that belong to the employed aptamer library. At varying extents, this problem occurs in all cultured cells and thus cannot be completely avoided[41]. This observation led us to develop an alternative approach to overcome this limitation. Here we describe our approach through a step-by-step detailed protocol on the setup, adjustment and conduct of a cell-targeting *in vitro* selection process. The protocol implements a fluorescent-activated cell-sorting (FACS) device to identify a certain cell population. The flow cytometer, therefore, simultaneously isolates the cell population of interest and removes unbound nucleic acid species. This procedure is, to the best of our knowledge, the first described that simultaneously identifies and eliminates cells from complex cell mixtures to enrich for cell-specific aptamers.

We applied this method in the selection of an ssDNA population that binds to vital CD19$^+$ Burkitt's lymphoma cells (CD19 is a B cell–specific surface marker protein), wherein remarkably only ten selection cycles were necessary[40]. The isolated aptamers distinguished between Burkitt's lymphoma B cells and primary B cells. Nonselected members of the initial ssDNA library did not exhibit any detectable specific binding to the target cells.

Our protocol is complementary to the one provided by Sefah *et al.* in an important respect: although the procedure from Sefah *et al.* might be more straightforward for adherent cells, the FACS-SELEX approach described here is the method of choice for cells that grow in suspension or for primary cell isolates such as those frequently obtained in the clinic. The ability to choose between the two protocols will enhance the success rate of cell-SELEX experiments, as exemplified by the direct comparison of the two approaches as described in Raddatz *et al*[40].

Our method is generally applicable to the screening of libraries of single-stranded DNAs, and also with slight modifications, such as *in vitro* transcription and reverse transcription that are described in detail elsewhere[27,42,43], to chemically modified RNAs containing 2′-fluoro-2′-deoxyribose. The method described here should work on any cell population for which specific staining antibodies or any other fluorescence-based labeling strategies (i.e., dead cell and/or living cell stains) are available and it could lead to individual aptamer sequences that recognize the cell population applied in the selection. The aptamers found may have potential as diagnostic tools or tools for biomedical research. They could also be used as precursors for the setup of aptamer-displacement screens to find small molecule lead structures that affect the target recognized by the aptamer and thus could serve as potential drugs. Moreover, this method may also be a first step toward the use of aptamers for individualized diagnostic and medical applications, because this selection process will be accessible to clinical laboratories for the assessment of high-affinity and specific cell-targeting agents that are promising diagnostic or biomedical research tools and thus is a useful complement to widely used antibody-based assays. 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 that are present in primary tissues or body fluids.

Our detailed protocol consists of three parts (**Fig. 2**). The first part (**Fig. 2**, green boxes) describes the steps that establish the nonspecific binding of the ssDNA library to the target cells, an inherent property of the employed nucleic acid library on which the subsequent design of the experiment crucially depends. It is equally important to ensure that the cell culture conditions and the integrity of the cells are maintained throughout the entire selection protocol. Furthermore, adjusting the optimal conditions for the amplification procedure of the selected nucleic acids after elution from the target

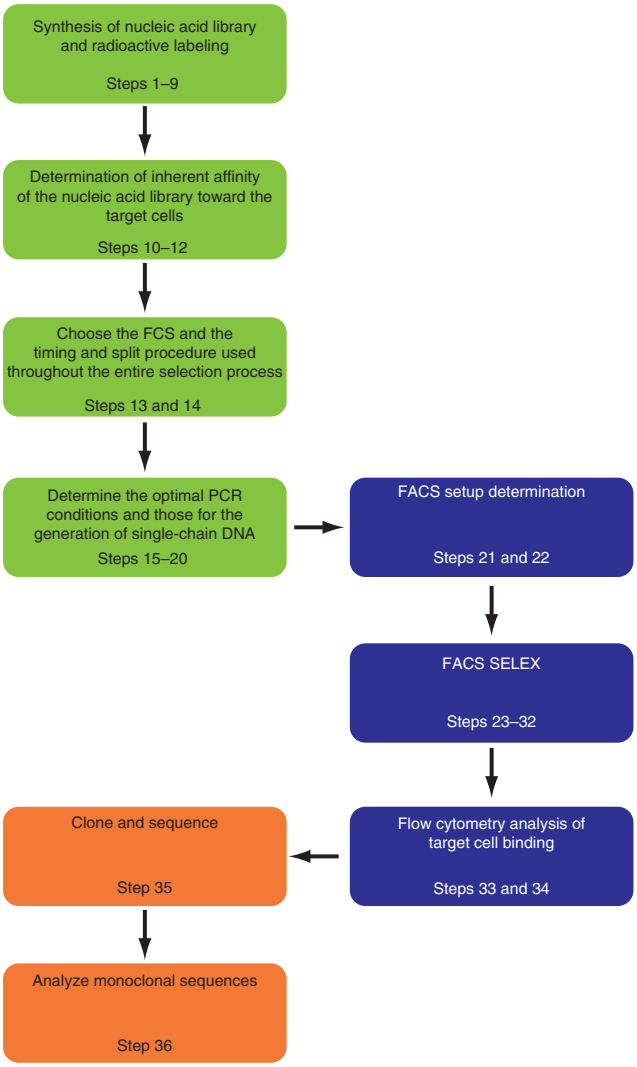

Figure 2 | Workflow for setting up and executing a FACS-SELEX experiment. The numbers refer to the numbered steps in the step-by-step protocol.

cells is a prerequisite for a successful FACS-SELEX procedure. In part two (**Fig. 2**, blue boxes), the actual FACS-SELEX process after adaptation of the optimal selection conditions to the flow cytometer is described. It is necessary to determine optimal cell staining conditions and to optimize the threshold and gain settings of forward and side scatter, as well as of fluorescence detectors. Part three (**Fig. 2**, orange boxes) describes the analysis and evaluation of the selected library and sequences. An overview of the entire process and its analysis is presented in the ANTICIPATED RESULTS section.

Experimental design strategy

Before starting with the FACS-SELEX approach, several prerequisites need to be addressed. These are summarized in **Box 1**, and more details are given in the following section.

FACS setup

In our laboratory, the initial display of data necessary for the purification of target cells (**Fig. 3**) was optimized for a FACSDiVa cell sorter (BD Bioscience), which represents a stream-in-air sorter; it should be modified for cell sorters from other manufacturers. However, the setup for the detection and sorting should be similar on a variety of cell sorters.

FACS and analysis of the data presented in **Figure 3** was performed using a solid-state laser for excitation of fluorescein-labeled ssDNA, emitting 150 mW at 488 nm. Forward scatter was collected on a photodiode through a 488/10-nm band-pass filter. An identical 488/10-nm band-pass filter was placed in front of the side scatter photomultiplier. Fluorescence emission from fluorescein-based probes was collected with a 530/20-nm band-pass filter. All pulses were displayed on a logarithmic scale to get the full dynamic range. Setup, optimization and monitoring of the cytometer settings were performed as recommended and described elsewhere[44,45].

Library design

The sequence and design of the applied ssDNA library is of importance for the success of the selection procedure. The annealing of both primer molecules to the template strand needs to be very efficient and specific, as many different nucleic acid molecules of cellular origin will be present during the amplification step of the library. General artifacts observed are (i) the amplification of nucleic acids even in the absence of the library (false positives), (ii) the suppression of amplification and (iii) the appearance of nucleic acid species with different lengths compared with the library. In addition to the library described in this protocol (D1 library), we have developed further libraries and primer pairs that are also suitable for cell-SELEX procedures. These libraries have been optimized for annealing temperature and for being less prone to amplification artifacts such as overamplification and the appearance of molecular parasites[46–48].

Primer pair sequences used were as follows: (i) 5′-C47: 5′-GGG AGAGGAGGGAGATAGATATCAA-3′, 3′-C20: 5′-GTCCTGTGG CATCCACGAAA-3′; (ii) 5′-forward: 5′-AGCATAGAGACATC

BOX 1 | OVERVIEW OF IMPORTANT NOTES BEFORE PERFORMING FACS-SELEX

1. Pay careful attention to ensure that the starting conditions for SELEX are met.
   - Check that you are able to see the cells properly in forward scatter and side scatter.
   - Check that the staining used to discriminate between live and dead cells is reliable.
   - The composition and characteristics of the biological sample should be stable. Importantly, there should not be too many dead cells (<2%). Check that there is no significant overlap between the emission spectra for the stains used for cell-surface markers, live-dead staining and DNA labeling.
2. In terms of biochemical techniques required to perform SELEX, you should first demonstrate that:
   - Your PCR is efficient, specific and reproducible.
   - You are familiar with the cell culture handling and work up steps (to produce input and perform purification).
3. Background conditions must be set (e.g., optimize conditions such that initial binding of the ssDNA library to the target cells is <2%) and reproducible.

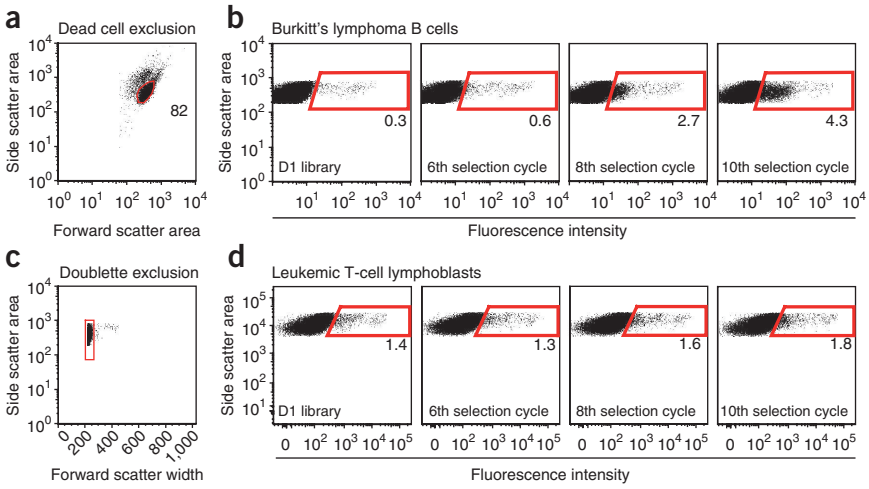

Figure 3 | Monitoring enrichment of ssDNA aptamers during FACS-SELEX. (**a**–**d**) The impact of dead cells on the selection process of cell type–specific aptamers can be excluded by setting a region of interest (**a**) in a forward scatter versus side scatter dot plot on membrane-intact cells. The position of live cells can be verified by staining a parallel sample with corresponding fluorochromes for live-dead discrimination (e.g., calcein AM). Aggregates should be further excluded by forward scatter width versus area parameters, as shown in **c**. (**b**,**d**) Cells present in the logical combination of these two regions were further displayed in a dot plot of side scatter versus fluorochrome intensity of the corresponding ssDNA dye labeling. Cells were repeatedly sorted on the region presenting cells that exhibit a positive staining for the incubated ssDNA. Successful enrichment of cell-type-specific aptamers can be monitored by an increasing percentage of cells present in the region of cells exhibiting increased binding of fluorescence-labeled aptamers to the target cells (**b**, Burkitt's lymphoma B cells), as compared with control cells (**d**, leukemic T-cell lymphoblasts). The corresponding part in the workflow shown in **Figure 2** is colored blue (Steps 33 and 34).

TGCTAT-3′, 3′-reverse: 5′-TACCTGAAGTCTGGAGTCTA-3′; (iii) 5′-D3: 5′-GCTGTGTGACTCCTGCAA-3′, 3′-D3: 5′-GGAGACAAGATACAGCTGC-3′.

PCR and generation of single-stranded DNA molecules

The amplification of the DNA library is a crucial step during the selection process. The optimized PCR protocol provides a size-homogenous double-chain DNA library that is used to extract the single-chain DNA molecules for the next selection cycle. To achieve this, various protocols have been developed, although the most common protocol uses biotinylated primer molecules to enable streptavidin-biotin chemistry and alkaline denaturation to separate the two strands. In this procedure, there is a risk that the single-chain DNA will be contaminated with streptavidin, which has been shown to have the potential to activate cells, thus hampering and modifying the addressed target cells[49]. Alternatively, a 3′ primer with a nonamplifiable extension, such as an 18-carbon-atom spacer (C18), can be used during the PCR. The separation of both strands can subsequently be achieved by denaturing polyacrylamide gel electrophoresis (PAGE). However, this approach requires a larger amount of ssDNA, as visualization by ultraviolet (UV) shadowing before cutting the correct-sized ssDNA is required. For this approach, we usually perform at least fifteen 100 µl PCR reactions. A suitable primer molecule that fits with the described D1 library (see REAGENTS) is given as an example below. This primer fits the ssDNA library D3 described above and bears a nonamplifiable extension (C18), followed by a poly-dA sequence and a 5′-terminal hairpin structure that induces slower migration on the PAGE gel, owing to secondary structure formation: 5′-GGGCGATCGTAAGATCGGCCCAAAAAAAAAAA-18C-GGGAGACAAGAATAAGCATG-3′.

Calcein AM staining of live cells

We identified the presence of dead cells or cells with reduced membrane integrity as problematic. These cells soak up DNA (and probably other nucleic acids as well) in an unselective manner. As these cells are present in varying amounts in almost all cultures and primary cell preparations, they prevent the enrichment of specific live cell–interacting aptamers. To rule out these cells, we performed a live-dead cell separation, enabled through visualizing those cells with an active metabolism with calcein AM.

Alternatively (or in addition), the protocol can be extended with specific antibody staining to further address an additional defined subpopulation of cells. For example, a fluorescently labeled antibody targeting CD19 has been applied to specifically address the B cell subpopulation in PBMCs (peripheral blood mononuclear cells)[40]. This fluorescent label can also be implemented in the FACS-SELEX approach. As many cell-specific markers and antibodies for them are available, this approach can be applied to various cells.

Simultaneous monitoring of selection progress

As the employed DNA library is labeled with fluorescein and separation of bound from unbound DNA is maintained by flow cytometry and cell sorting, the course of the selection can be monitored simultaneously. Thus, no additional assays are required to investigate the binding behavior of the libraries. This represents an advantage of the FACS-SELEX procedure; it has the further advantage of avoiding the extensive use of radioactivity.

Cell culture maintenance and incubation

The maintenance and treatment of the targeted cells is of utmost importance for the success of the selection. We strongly recommend verifying the identity of the cells used before selection. The applied procedures to split, grow and handle the cells should never be changed. In addition, the FBS used for cell growth should not be changed during the selection process, as different FBS batches can have tremendous effects on the cell cycle and the presentation of molecules on the surface of cells. The incubation time of the DNA library with the target cell should also be strictly kept constant. We decided to use the cells at 37 °C, as this best reflects the *in vivo* situation. However, cells cannot be considered as static molecules and, with respect to a consistent selection process, the incubation time should be as short as possible to avoid fluctuations in the cell surface presentation of potential target molecules. Our protocol uses an incubation time of 30 min. Alternatively, it might possible to incubate at 4 °C, thus reducing metabolism and turnover of cell surface presentation of molecules. However, it is of risk to identify aptamers that bind to their respective target molecules only at 4 °C, but not at temperatures that reflect physiological conditions[50].

MATERIALS

REAGENTS

- Taq DNA polymerase (5 U μl^{-1};Promega)
- Taq reaction buffer (10×; Promega),
- $MgCl_2$ solution, 25 mM
- dNTP mix (10 mM each; Roche)
- Synthetic oligodeoxynucleotides: ssDNA library (D1: 5′-GCCTGTTGTGA GCCTCCTAAC-N49-CATGCTTATTCTTGTCTCCC-3′), 5′-D1 primer (5′-GCCTGTTGTGAGCCTCCTAAC-3′), 3′-D1 primer (5′-GGGAGACA AGAATAAGCATG-3′)
- Polynucleotide kinase (PNK) buffer, T4 PNK (10×; Promega)
- γ^{32}P-ATP (Perkin-Elmer) **! CAUTION** Special treatment procedures, such as plexiglass protection shields and storage boxes need to be used when working with ^{32}P-isotopes.
- MicroSpin gel filtration G-25 columns (GE Healthcare)
- Phosphate-buffered saline (PBS)
- Hank's balanced salt solution (HBSS)
- Streptavidin-coated beads (Dynabeads M-270, GE Healthcare)
- RPMI 1650 with L-glutamine (PAA)
- Fetal bovine serum (FBS; Invitrogen)
- Penicillin-streptomycin (Invitrogen)
- Bovine serum albumin, nuclease free (BSA; Calbiochem)
- Salmon sperm DNA (Invitrogen)
- Target cell lines or primary cells (Burkitt's lymphoma cells, DSMZ, cat. no. ACC 603)
- Phenol (Roth)
- Chloroform (Riedel-de-Haën)
- Ethanol (Roth)
- Heparin
- Antibodies (anti-CD19$^+$-PE labeled, Becton Dickinson)
- Distilled water (dH_2O)
- Sodium chloride
- Tris-HCl
- EDTA
- Urea
- Bromphenol blue
- Xylencyanol blue
- Sodium acetate
- Sodium hydroxide
- Hydrochloric acid
- Calcein AM stain

EQUIPMENT

- Nuclease-free working environment
- Thermocycler
- Water bath, thermal block
- UV spectrophotometer
- Agarose gel electrophoresis equipment
- Gel documentation system
- Polyacrylamide gel electrophoresis equipment
- Phosphorimager (Fuji FLA3000, Fujifilm)
- Scintillation counter
- Microcentrifuge
- Centrifuge for FACS tubes (temperature controlled)
- Cell culture equipment
- Silanized, sterile glass wool
- Equipment for cloning and sequencing of DNA library
- *Flow cytometry equipment*: analyzer (BD LSR II, BD Biosciences) and cell sorter (BD FACSDiVa, BD Biosciences)

REAGENT SETUP

Bind & wash buffer (B&W buffer; 2×) Combine 2 M NaCl, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. A volume of 250 ml is sufficient for this protocol. The buffer can be stored at room temperature (20 °C) for up to 3 months.

PAGE loading buffer Combine 9 M urea and 50 mM EDTA in dH_2O. A volume of 250 ml is sufficient. The buffer can be stored at room temperature for at least 3 months.

PAGE marker PAGE marker is PAGE loading buffer supplemented with a spatula tip of bromphenol blue and xylencyanol blue. 250 ml is sufficient. The buffer can be stored at room temperature for up to 6 months.

SELEX buffer SELEX buffer is composed of either PBS or HBSS containing 1.4 mM $MgCl_2$, 1 $\mu g\ \mu l^{-1}$ BSA and 1 $\mu g\ \mu l^{-1}$ salmon sperm DNA. Both PBS and HBSS are commercially available and can be stored at room temperature for up to 6 months. The other ingredients must be added fresh before use.

PROCEDURE

Determination of background binding of initial ssDNA library ● TIMING 2 d

1| Perform 5′-radioactive labeling of the synthetic ssDNA library using T4 PNK and γ^{32}P-ATP. Prepare the solution as shown in the table below, and incubate it for 45 min at 37 °C. Add 120 µl dH_2O.

Reagent	Volume (µl)	Final concentration
PNK buffer (10×)	4	1×
ssDNA (20 µM)	1	0.5 µM
T4 PNK (10 U)	4	40 U
γ^{32}P-ATP	4	0.5 µM
H_2O	Add to make up to 40 µl final volume	

! CAUTION Follow appropriate safety procedures for working with radioactivity.

2| Remove nonincorporated γ^{32}P-ATP by gel filtration using G-25 spin columns (remove storage buffer by spinning at 420*g* for 1 min, then load sample and spin for 2 min at 420*g*).

3| Precipitate the filtrated ssDNA by adding one-tenth volume of 3 M sodium acetate (pH 5.4) and three volumes of absolute ethanol; incubate at −80 °C for 10 min.

4| Centrifuge the precipitated ssDNA in a cooled (4 °C) microcentrifuge at 20,000*g* for 20 min.

5| Remove and discard the supernatant carefully. Wash the pellet with 70% (vol/vol) ethanol, remove the ethanol completely and dry the pellet in air. Resuspend the dry pellet in 50 µl PAGE loading buffer.

6| Further purify the reaction product by denaturing PAGE. To purify the 90 nucleotides comprising the D1 ssDNA library, we employed a 10% (wt/vol) polyacrylamide gel. For nucleic acids of different lengths, the percentage of the polyacrylamide gel needs to be adjusted accordingly. Heat the samples for 1 min at 95 °C before loading them on the gel.
? TROUBLESHOOTING

7| Prerun the gel at 370 V for at least 30 min. Flush the wells with a syringe before loading the samples. After running the gel for 1 h at 370 V, the bands can be visualized by autoradiography.
? TROUBLESHOOTING

8| Cut out the right-sized band with a sterile scalpel blade and recover the ssDNA by passive elution in 0.3 M sodium acetate (pH 5.4) at 65 °C for 1.5 h on a thermal shaker. To remove gel pieces, the mixture should be filtrated through sterile silanized glass wool.
? TROUBLESHOOTING

9| Precipitate the labeled ssDNA with standard ethanol precipitation (see Step 2) and dissolve the dry pellet in dH_2O.
■ **PAUSE POINT** The ssDNA can be stored at −20 °C for up to 6 months and at −80 °C for up to 2 years without loss of activity.
? TROUBLESHOOTING

10| Incubate the radioactively labeled ssDNA library (final concentration of 0.5 nM) with 1×10^6 target cells at 37 °C in a incubation volume of 1 ml PBS (supplemented with a final concentration of 1.4 mM $MgCl_2$) and in the presence of varying concentrations of competitors (as indicated in **Figure 4**) for 30 min.

11| Separate the supernatant from the cells by centrifugation at 200*g* for 5 minutes (fraction 1). Wash and centrifuge the pellet three times with 1 ml PBS (supplemented with 1.4 mM $MgCl_2$) and save each supernatant (fractions 2–4). Finally, resuspend the cell pellet in 1 ml PBS supplemented with 1.4 mM $MgCl_2$ (fraction 5).
? TROUBLESHOOTING

12| Count all fractions with the scintillation counter and determine the amount of radioactivity found in each fraction. Calculate the amount of ssDNA bound to the cells (Bkg) according to the formula below.

$$\text{Bkg} = \frac{\text{radioactivity in fraction 5}}{\sum_{i=1}^{5} \text{radioactivity in fraction } i} \times 100$$

▲ **CRITICAL STEP** The determination of the background-binding capacity of the ssDNA library to the target cells is crucial for the success of the selection procedure. Try different competitors, such as salmon sperm DNA, heparin or tRNA (choose one that does not interfere with cell signaling). The background-binding activity of the starting library should not exceed 1–2%. Otherwise, the selection process will be inefficient and causes either an enhanced number of selection cycles required for enrichment or a failure of the entire selection, owing to unspecific ssDNA recovery during the separation step of the selection procedure.

Cell culture maintenance ● TIMING Variable; occurs throughout the entire selection process

13| Culture Burkitt's lymphoma cells in RPMI 1640 medium (with L-glutamine) supplemented with 10% heat-inactivated FBS and 100 IU ml^{-1} penicillin-streptomycin. Adapt these conditions to those required by your cell line of interest.
▲ **CRITICAL STEP** For one entire selection experiment (SELEX and analysis), the same batch of FBS and the same heat inactivation procedure should be employed. Avoid

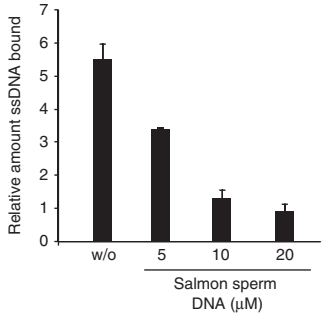

Figure 4 | Determination of the relative amount of ssDNA library bound to Burkitt's lymphoma cells in the absence (w/o) and presence of increasing amounts of competitor (salmon sperm DNA). The corresponding part in the workflow shown in **Figure 2** is colored green (Steps 10–12).

the introduction of changes to the cell culture maintenance procedure. The timing of the split procedures and SELEX thereafter should be kept similar during the entire selection procedure.
▲ CRITICAL STEP Frozen and rethawed cells should be cultured for at least 14 d before being used for the SELEX experiment.

14| Isolate PBMCs from the blood of healthy volunteers using standard procedures employing Ficoll gradient centrifugation to removing erythrocytes, granulocytes and platelets.
! CAUTION Special safety issues (i.e., vaccination) and ethical issues are to be considered. Blood from volunteers must be considered as potentially contaminated with life-threatening pathogens, and thus careful treatment of the samples and virus testing is recommended. Government restrictions and permission must be considered before taking the blood.
■ PAUSE POINT PBMCs can be stored before use in liquid nitrogen for up to 10 years. Check the quality of your samples before freezing and after thawing (e.g., with trypan blue).

Amplification and strand displacement ● TIMING 0.5 d
15| *PCR without cell extracts*: optimize the PCR conditions with regard to library and primer design, annealing temperature, amount of required PCR cycles, and PCR using modified primers such as fluorescently labeled and biotinylated primers.
▲ CRITICAL STEP All synthetic oligodeoxynucleotides must be lyophilized and HPLC and PAGE purified.
? TROUBLESHOOTING

16| Perform PCR with cell extracts (after optimization without cell extracts in Step 15).
▲ CRITICAL STEP Ensure that no amplicons can be detected when only cell extract (product of Step 16) is used as a template.
▲ CRITICAL STEP Ensure that extracts do not inhibit the PCR efficiency and do not interfere with the specificity of the PCR.

17| *PCR during the selection:* set up the PCR mixture and heat it at 95 °C for 5 min before performing the PCR program. (The parameters shown in the tables below are those optimized for the D1 ssDNA library and its respective primer pairs.)

Reagent	µl	Concentration
Taq reaction buffer (10×)	10	1×
$MgCl_2$ (25 mM)	8	2 mM
dNTPs (10 mM)	2	200 µM
5′-D1 primer (5′-fluorescently labeled) (100 µM)	1	1 µM
3′-D1 primer (5′-biotinylated) (100 µM)	1	1 µM
Taq DNA polymerase (5 U $µl^{-1}$)	6	2.5 U per 100 µl
Purified ssDNA template in H_2O	100–150	
H_2O	To give 1,200 µl of final volume	

PCR program	
Denaturation	60 s at 95 °C
Annealing	60 s at 60 °C (depending on primary sequence of the primers)
Extension	95 s at 72 °C

18| Analyze the PCR product by agarose gel electrophoresis (visualize with UV and ethidium bromide staining or any other suitable method)
▲ CRITICAL STEP Avoid exposure of PCR reactions to light.
■ PAUSE POINT Products of PCR reactions (double-stranded DNA (dsDNA)) can be stored at −20 °C for at least 2 months and at −80 °C for up to 2 years.

19| *Strand displacement:* incubate the reaction mixture prepared as shown in the table, for 30 min at room temperature in a head-to-tail shaker. Discard the supernatant after incubation and wash the beads two times with 1× B&W buffer and then once with 2× B&W buffer. The desired ssDNA is obtained by alkaline denaturation (150 mM NaOH), followed by neutralization with 100 mM HCl. Remove the salts from the sample with gel filtration (MicroSpin G-25 column).

Reagent	µl
PCR product	1,200
2× B&W buffer	1,200
Streptavidin-coated beads (5 mg in 1× B&W buffer)	2,400

20| *Product control*: analyze the fluorescently labeled ssDNA for purity (length) and yield by ethidium bromide–free (ethidium bromide might interfere with readout) agarose gel electrophoresis (read out with Fuji FLA3000). Determine the yield additionally with a UV spectrophotometer at 260 nm.

FACS setup: compensation and background control ● TIMING 0.5 d

21| Set up the FACS machine. The setup required for each particular instrument varies among the different cell sorters on the market. However, a panel of setup controls specific for the fluorochromes used is required for each experiment. As an initial step, preliminary measurement on stained samples versus unstained controls should be performed to optimize the threshold and gain settings on forward and side scatter, as well as on fluorescence detectors. Dyes should be chosen such that fluorescence can be excited and recorded individually, thereby minimizing spectral spillover. Sensitivity of detectors and compensation of fluorochrome emission overlap must be addressed in a specific order[44,45] and manufacturers now offer corresponding sets of calibration beads and software packages to address this issue. In a similar way, reproducibility and performance of your particular instrument can be monitored by custom combinations of calibration beads and analysis strategies or quality control reagents offered by the manufacturers[44,45].

▲ **CRITICAL STEP** Perform preliminary experiments for each dye with the cells of interest and the same batch of antibodies employed in the FACS-SELEX.

▲ **CRITICAL STEP** In each selection cycle, unstained target cells as well as target cells stained with fluorescently labeled ssDNA library should be used as control samples. Monitor and adjust compensation, threshold and gain settings.

22| To verify the results of the radioactive background determination and to prove their translation to the cytometer, the background binding to target cells is also determined by flow cytometry. Therefore, the starting library (D1) has to be fluorescently labeled, e.g., with fluorescein. Perform the general selection protocol (see Steps 23–27) using varying amounts of competitors (e.g., 0.1–1.5 µg µl^{-1} salmon sperm DNA; 0.05–0.5 µg µl^{-1} BSA; 1–10 µM tRNA; or 0.01–1 µg µl^{-1} heparin).

▲ **CRITICAL STEP** Competitors should be stable under selection conditions; for this reason, tRNA might be the last choice, as ribonuclease activity of biological samples might lead to fast degradation.

▲ **CRITICAL STEP** Be sure to get a clear picture about the binding behavior of the fluorescently labeled ssDNA library toward different cell populations (i.e., 'vital cells' compared with 'cells with compromised cell membranes' in the simplest case; however, binding behavior might also depend on the cell type). Choose competitor conditions that lead to a strongly reduced background binding (e.g., ≤2% of input ssDNA remains on the cells; no significant shift is observed in the fluorescently stained–ssDNA histogram compared with nonfluorescently stained cells). Adjust the SELEX conditions to support your specific experimental needs.

▲ **CRITICAL STEP** There are only limited suitable fluorescent dyes that are cost effective, routinely used and available for synthetic nucleic acid libraries: fluorescein, fluorescein isothiocyanate, rhodamine, TAMRA (tetramethyl-6-carboxyrhodamine) and the Alexa Fluor family of dyes. The choice of the dye also depends on the configuration of the cytometer used for analysis and cell sorting.

▲ **CRITICAL STEP** Any dye chosen should not interfere with the binding behavior in the selection step or with the ability to amplify the obtained enriched libraries enzymatically through PCR. Avoid choosing dyes that might have an impact on your ssDNA, such as those involved in folding or those that are large, thus facilitating steric hindrances. One such dye might be phycoerythrin.

▲ **CRITICAL STEP** Leaky and dead cells interfere with the selection process and must be excluded during cell purification by FACS. Dead cells can be identified by altered light scatter characteristics (see **Fig. 3**), especially in nearly homogenous cell populations such as cell lines. More complex cell populations may require an additional staining for cells with damaged membranes. Any dye used should not interact with nucleic acids. Dyes such as propidium iodide (DNA intercalation) and DAPI (4,6-diamidino-2-phenylindole) or bis-benzimide H33258 (DNA minor groove binding), which are commonly used for live-dead discrimination, are therefore less well suited (i.e., you should not use intercalators such as propidium iodide). Alternative fluorochromes that display intracellular enzyme activity, such as fluorescein diacetate or calcium indicators can be used to identify live cells. However, it must be certified that the spectra do not interfere with the fluorochrome used for labeling the ssDNA.

FACS-SELEX ● TIMING 2–3 d per cycle

23| *First selection cycle*: Incubate 1× 10^6 target cells with 500 pmol of the ssDNA library in 1 ml SELEX buffer at 37 °C for 30 min.

24| First, label a control sample of cells with your preferred dye for live-dead discrimination. We employed calcein AM to visualize live cells simultaneously with the incubation of the nucleic acid library. The following scheme was applied:

700 µl	Burkitt's lymphoma cells (~1 million in HBSS with $MgCl_2$)
10 µl	BSA (10µg $µl^{-1}$)
99 µl	Salmon sperm DNA (10.1µg $µl^{-1}$)
15 µl	$MgCl_2$ (10 mM)
30 µl	10× HBSS
10 µl	Calcein AM
50 µl	DNA library (1 µM)
86 µl	H_2O

25| Run the sample on the cell sorter and record a sufficient number (10,000–20,000) of events to characterize live and dead cells in your sample. Identify these cells in a two-parameter display—forward scatter versus side scatter (**Fig. 3a**).

26| Set a region of interest on the cells in the scatter parameter display that corresponds to live and intact cells. Set a second region of interest in the forward scatter area versus forward scatter width that includes single cells and excludes aggregates (**Fig. 3c**).

27| Display the fluorescence of the labeled cells, which are present within these two regions, in a separate dot plot (e.g., side scatter versus ssDNA fluorochrome) and define the population of positive (live) cells. Follow the manufacturer's instructions to optimize the sorting conditions for purity. Optionally, a counter-SELEX step with other cells can be included to eliminate general cell-binding sequences.[39,51]

▲ **CRITICAL STEP** Perform all centrifugation and washing steps at 37 °C.

▲ **CRITICAL STEP** Filter the cells through gauze (40 µm) before flow cytometry to avoid clogging the cytometer.

28| Recover the isolated ssDNA molecules from your sample by heat elution/denaturation (add 100–150 µl dH_2O and heat for 5 min at 95 °C). Purify from further contaminants by phenol-chloroform extraction and gel filtration (MicroSpin G-25 columns).

29| Amplify the purified ssDNA by PCR. The input ssDNA for the second cycle is generated from the double-stranded PCR product by strand displacement (see also Steps 17 and 19 for details).

? TROUBLESHOOTING

30| The yield and integrity of the generated ssDNA can be analyzed by agarose or polyacrylamide gel electrophoresis (ethidium bromide–free, Fuji FLA3000) and UV spectrometry at 260 nm.

▲ **CRITICAL STEP** Protect fluorescently labeled DNA samples from exposure to light.

31| *Second selection cycle*: from the second cycle onward, 50 pmol of ssDNA is used. Optionally, additional washing steps can be introduced after the incubation step, whereas separation of bound from nonbound ssDNA can be achieved by mild (200*g*) centrifugation. The *g*-value needs to be carefully adjusted for every cell type used. Too-harsh centrifugation (*g*-values > 500) may result in an enhanced number of dead cells, thus counteracting the selection process. Dead cells can be discriminated from live cells by calcein AM staining.

? TROUBLESHOOTING

32| Continue the selection until a substantial shift in binding of the ssDNA toward the target cells is observed.

▲ **CRITICAL STEP** Assure that the cells are always under comparable conditions (e.g., always take confluent cells), as the selection targets might be expressed in different concentrations on the cell surface, depending on cell culture conditions.

■ **PAUSE POINT** After each selection cycle, at the level of PCR products (dsDNA), dsDNA can normally be stored for at least 2 years at −20 °C without loss of activity.

Analysis of the selection ● TIMING ~1 month

33| Validate the binding of selected ssDNA libraries, compared with the starting ssDNA library toward the target cells, using flow cytometry.

34| Analyze the specificity of the enriched libraries for the target cells by including various cell lines or primary cells as positive or negative controls in flow cytometry studies.

35| Clone and sequence the enriched library.

36| Analyze monoclonal sequences for binding and specificity toward target cells using flow cytometry. Alternatively, radioactivity-based methods can be applied. However, these methods do not enable the discrimination between live and dead cells.

▲ **CRITICAL STEP** Always determine the concentration and the labeling of ssDNAs being used directly before experiments.

? TROUBLESHOOTING

Step 6: To increase the solubility of ssDNA before PAGE purification, you can dissolve the pellet in a small amount of water and subsequently add the PAGE loading buffer. The yield of ssDNA after PAGE purification is 50–70%.

Steps 7 and **8:** Running time of 1 h is optimal for our DNA library. When using other libraries, this value may need to be adjusted.

Step 9: The washing step of the radioactive DNA pellet needs to be carried out very carefully to avoid the loss of selected DNA sequences. If the pellet does not dissolve quantitatively in dH_2O, heat the sample carefully.

Step 11: Remove the supernatant carefully to avoid loss of cells. If cell pellets are unstable or hardly visible, we recommend using FACS tubes instead of standard microliter tubes. Avoid high *g*-values during centrifugation, as these cause damage to cells.

Step 15: If the cell extract forms an amplicon in the absence of library template, the primer design must be reassessed. The purification protocol after separation should also be reassessed if an increased number of PCR cycles are required (or if a complete inhibition of amplification is observed). Critical questions to be taken into account are as follows: was the interphase of the phenol-chloroform extraction fully removed? Was only the watery phase recovered—no phenol or chloroform entered the PCR? Are phenol-chloroform extraction and gel filtration sufficient in the specific setup or is there a need for additional purification?

Step 29: If no amplified dsDNA is observed, we recommend performing the selection cycle again. Amplification procedures should be kept constant throughout the entire selection process. If, unexpectedly, many more (>3) selection cycles than estimated are required to obtain a proper dsDNA band on the agarose gel, some steps during the selection cycle were inaccurate. For example, phenol must be removed quantitatively, as it inhibits polymerase activity. Always double-check the quality and performance of the PCR master mix by including positive and negative controls during PCR amplification after each selection cycle. If DNA isolation and amplification after the elution step is not fast enough, DNA may be degraded. If this occurs, redo the selection cycle. If amplicons of different sizes appear, which is a strong indicator that so-called 'molecular parasites' are enriched, it is sometimes useful to include a gel purification step before starting the next selection cycle. However, frequently these parasites cannot be avoided because of superior replication properties. In this case, change the DNA library and use new primer pairs. Suboptimal amplification behavior is a strong hindrance to the success of a cell-SELEX approach.

Step 31: If you choose to label the starting library with a fluorescent dye, do not expect to observe a big shift in fluorescence compared with the ssDNA-free negative control in the first cycle(s). Remember that the background was adjusted in a way such that that only a small amount of the library is supposed to bind to the cell surface.

● TIMING

Steps 1–12, Determination of background binding of initial ssDNA library: 2 d

Steps 13 and 14, Cell culture maintenance: variable; occurs throughout the entire selection process

Steps 15–20, Amplification and strand displacement: 0.5 d
Steps 21 and 22, FACS setup: 0.5 d
Steps 23–32, FACS-SELEX: 2–3 d per cycle
Steps 33–36, Analysis of the selection: ~1 month

ANTICIPATED RESULTS

Before conducting the selection, all individual stages of the SELEX procedure need to be optimized and shown to be dependable. The main pre-SELEX experiments are the determination of the background-binding ability of the ssDNA library to the target cells and adjustment of purification followed by PCR amplification procedures. The former can be addressed by performing a radioactive assay employing ^{32}P-labeled ssDNA library (Steps 1–9). Labeled ssDNA is incubated with the target cells (1×10^6), and after incubation the cells are pelleted and the supernatant removed and kept on ice. After washing the pellet, all fractions are counted with a scintillation counter and the amount of ssDNA retained on the target cells are calculated. The influence on target cell binding of different concentrations of a competitor molecule, such as salmon sperm DNA, can thus be elucidated. **Figure 4** shows that addition of 10 μM salmon sperm DNA to the incubation medium for Burkitt's lymphoma cells is sufficient to reduce the nonspecific binding of the ssDNA library to acceptably low levels.

While performing a successful SELEX, one will observe the increase in the percentage of cells that bind the selected ssDNA library compared with the starting library. We carried out ten rounds of *in vitro* selection targeting vital Burkitt's lymphoma cells. Our enriched nucleic acid library revealed enhanced binding to the target cells. In general, one should neither require a high number of selection cycles nor observe selection artifacts. Artifacts, such as molecular parasites or primer dimers, can be recognized by their sizes in analytical agarose gel electrophoresis; nucleic acids that are smaller or larger, as compared with the starting library, often represent selection artifacts.

Following selection, we have subsequently cloned and sequenced the enriched nucleic acid library.[40] The analysis revealed a high sequence diversity of the selected library compared with libraries obtained by selection against homogenous targets; however, the selected library contained several monoclonal DNA sequences that can be grouped into distinct sequence families. This is consistent with the collection of diverse potential target molecules present on the cell surface and with our aim to gain enriched libraries that contain different aptamers targeting various cell-surface structures. The library can contain aptamer species varying in binding intensity and specificity. One representative aptamer sequence (C10) was further analyzed. The characterization may include, among other things, the determination of a dissociation constant toward the target cells and specificity testing of control cell lines.

The impact of dead cells on the selection process of cell type–specific aptamers can be excluded by setting a region of interest in a forward versus side scatter dot plot on membrane-intact cells. The position of live cells can be verified by staining a parallel sample with corresponding fluorochromes for live-dead discrimination. Aggregates should be further excluded by forward scatter width versus area parameters, as shown in **Figure 3c**. Cells present in the logical combination of these two regions were further displayed in a dot plot of side scatter versus fluorochrome intensity of the corresponding ssDNA dye labeling (**Fig. 3b** and **3d**). Cells were repeatedly sorted on the region representing cells that exhibit a positive staining for the incubated ssDNA. Successful enrichment of cell type–specific aptamers can be monitored by an increasing percentage of cells present in the region of cells exhibiting increased binding of fluorescence-labeled aptamers to the target cells (Burkitt's lymphoma B cells), as compared with control cells (leukemic T-cell lymphoblasts).

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