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Efficient Selection of Silenced Primary Cells by Flow Cytometry

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• Abstract

RNA interference has emerged as a new and potent tool to knockdown the expression of target genes and to investigate their functions. For short time experiments with mammalian cell lines, RNA interference is typically induced by transfecting small interfering RNAs (siRNAs). Primary cells constitute important experimental systems in many studies because of their similarity to their in vivo counterparts; however, transfection of these cells has been found to be difficult. As a consequence, RNA interference of primary cells may result in mixed phenotypes because of the simultaneous presence in the same preparation of transfected and nontransfected cells. This may be particularly inconvenient when certain experiments (for example, biochemical analysis) should be performed. We use fluorescently labeled siRNAs to induce RNA interference in fibroblasts, and flow-cytometry associated cell sorting to separate subpopulations of transfected cells according to fluorescence intensity. Flow cytometry allows one to discriminate between strongly- and weakly- or nonsilenced fibroblasts, since the fluorescence intensity of transfected cells is related to the number of internalized siRNA copies and to the mRNA knockdown efficiency. The use of fluorescently labeled siRNAs may allow one to isolate by flow-cytometry associated cell sorting the most efficiently silenced primary cells for subsequent analysis.

• Key terms

RNAi; fluorescent siRNAs; mRNA knocking-down in fibroblasts

RNA interference (RNAi) is a posttranscriptional, highly conserved process in eukaryotes that leads to specific gene silencing (1–3). Double-stranded RNAs are processed by an enzyme called Dicer to generate duplexes of about 21 nt (short interfering RNAs, siRNAs) which mediate sequence-specific mRNA degradation. In mammalian cell lines, RNAi may be triggered by synthetic siRNA molecules or by plasmid and viral vectors designed to express short hairpin RNA (shRNA) molecules, which are processed by the cellular machinery to the corresponding siRNAs (4–6). In both cases, gene silencing results from destruction of mRNA that is complementary to the siRNA molecules without induction of the nonspecific interferon response pathway (7,8). Thus, silencing of gene expression by RNAi is a powerful tool to analyze the function of mammalian genes.

Although satisfactory RNAi results are obtained by introduction of siRNAs into many cell lines, with some others (9), particularly primary cells (10), siRNA delivery remains as one of the major constraints of this approach. In hard-to-transfer cell types, like primary cells, RNAi may result in a mixed phenotype, milder than that exhibited by null mutants or efficiently transfected cells, due to the coexistence of silenced and nonsilenced cells in the experiments. To improve the transfection efficiency, several procedures have been developed, including the use of retroviral vectors and electroporation. However, these procedures have some disadvantages. Thus, electroporation may increase cell mortality, because of membrane damage and ionic

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imbalance, and, in addition, some cells are electroporation-resistant (11,12). The use of retroviral vectors for production of siRNAs allows one to select the transfected cells with antibiotics, thus avoiding the problem of a mixed phenotype. However, transduction with retroviral vectors is difficult in some cases, since they require active cell division for gene transfer. Although this problem can be overcome with lentiviral vectors, which are able to mediate gene transfer in nondividing cells (13,14), the oncogenic potential of retrovirus (9,15,16) should not be neglected.

A different approach to improve RNAi results is based on the physical separation of transfected from nontransfected cells. Thus, cotransfection of plasmids encoding shRNAs with a plasmid encoding either CD19 or CD20 surface marker proteins, followed by immunopurification of CD19- or CD20-expressing cells, was shown to be a worthwhile approach in cells transfected with low efficiency to separate the minor population of transfected cells from the rest (17). In this regard, it is interesting to note that flow cytometry allows one to identify and to isolate cells from an heterogeneous population on the basis of specific properties such as multiple fluorescences (18,19). The delivery of fluorescently labeled siRNAs into the cells may be monitored by flow cytometry; in fact, fluorescently labeled siRNAs are frequently used as markers when optimising transfection conditions. Thus, we reasoned that the combined use of fluorescently labeled siRNAs and flow-cytometry associated cell sorting might be a convenient approach to separate transfected from nontransfected primary cells. In this work, we show that flow cytometry allows one to discriminate between strongly- and weakly- or nonsilenced fibroblasts, since the fluorescence intensity of transfected cells is related to the number of internalized siRNA copies and, thus, to the mRNA knockdown efficiency.

Materials and Methods

Labeling of Double Stranded siRNA

Both negative control and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) chemically synthesized double stranded siRNAs were labeled with the Silencer Cy5 Labeling Kit (Ambion, Austin, TX). Briefly, 5 μg of the negative control and GAPDH siRNAs were incubated with 7.5 μl of the Cy5 labeling reagent for 1 h at 37 °C in the dark. After labeling, the siRNAs were precipitated and resuspended according to the manufacturer's instructions. 6-carboxy-fluorescein phosphoramidate (FAM)-labeled GAPDH siRNA was provided by Ambion.

Cell Culture and Transfections

HEK-293 cells (human embryonic kidney, ATCC CRL-1573) and 3349B normal human fibroblasts (Coriell Institute for Medical Research, Camden, NJ) were cultured in modified Eagle's medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin (Gibco Life Technologies, Grand Island, NY). 2×10^5 cells were plated in 9.5 cm^2 culture dishes containing 10 mm diameter glass cover slips. Transfections with labeled siRNAs (final concentration in the culture media: 100 nM) were performed 24 h after plating, using 10 μl of siPortAmine (Ambion), unless otherwise indicated, according to the manufacturer's protocol.

Fluorescence Microscopy

After 72 h posttransfection, cells grown in coverslips were fixed with 3% paraformaldehyde (Sigma-Aldrich) for 30 min. The transfection efficiency was analyzed by direct fluorescence, using an Axioscope 2 microscope (Zeiss, Thornwood, NY). Images were taken with a CoolSNAP Fx digital camera and edited with the RSI image software (Photometrics, Roper Scientific, Tucson, AZ).

Total RNA Extraction and Analysis by RT-PCR

Cells were harvested using trypsin-EDTA (Sigma-Aldrich) and centrifuged at 100*g* for 10 min. Total RNA was extracted using an RNA extraction kit (GeneElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich). GAPDH mRNA was measured by quantitative one step RT-PCR, using specific oligonucleotides, Sybr Green dye, and a Gene Amp 5700 Sequence Detection System (Applied-Biosystems, Foster City, CA). Expression levels were normalized against cyclophilin as an endogenous control. The specific oligonucleotides used were: GAPDH-forward primer, 5′-TGGCCTACACTGAGCAC CAG-3′; reverse primer, 5′-GGGTGTCGCTGTTGAAGTCA-3′; cyclophilin-forward primer, 5′-CAAATGCTGGACCAAACA CAA-3′; reverse primer: 5′-GCCATCCAGCCACTCAGTCT-3′ (Sigma-Aldrich). Relative expressions were calculated using the comparative delta C_T method.

Flow Cytometry and Cell Sorting

Flow cytometry analyses of silenced cells were performed in a Cytomics-F500 flow cytometer coupled to the Cytomics RXP software (Beckman-Coulter, Brea, CA). Dead cells were excluded by their morphological features as displayed in dot-plots of forward scatter (FS, a rough estimation of cell size) versus side scatter (a rough estimation of cell granularity). Cells transfected with Cy5-labeled GAPDH siRNA were

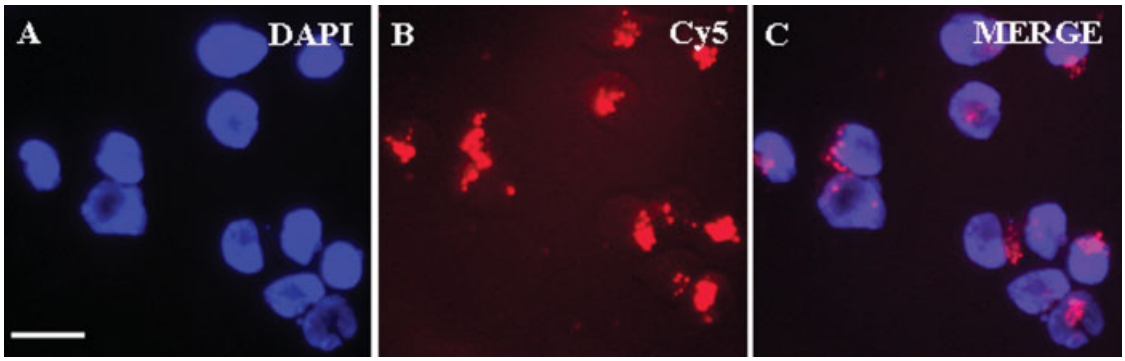

Figure 1. Fluorescence microscopy of cells transfected with Cy5 labeled siRNA. HEK-293 cells were transfected with GAPDH siRNA labeled with Cy5 and analyzed by fluorescence microscopy. Staining with DAPI of nuclear DNA (**A**) and Cy5 (**B**) are merged in panel (**C**). Bar: 25 μm.

detected as being positive for red fluorescence (FL4, 675 ± 25 nm). Cells transfected with FAM-labeled GAPDH siRNA were identified as positive for green fluorescence (FL1, 525 ± 20 nm). To separate physically FAM-GAPDH siRNA transfected cells, a MoFlo High-Performance Cell Sorter was used (Dako, Glostroup, Denmark). After sorting, cells were centrifuged at 100*g* for 10 min and total RNAs were extracted for quantitative one step RT-PCR measurements.

Results and Discussion

To determine the most efficient conditions under which flow cytometry may discriminate cells for fluorescence associated to internalized siRNAs, we first used HEK-293 cells. Different conditions of transfection of HEK-293 cells with a GAPDH specific Cy5-labeled siRNA were tested and the results were analyzed in a fluorescence microscope. Under the conditions described in the Materials and methods section and using 10 μl of siPortAmine, most cells contained the Cy5-labeled siRNA (Fig. 1), and the amount of GAPDH mRNA in transfected cells, calculated by real time PCR, was about 25% of that found in nontransfected cells (data not shown).

Next, HEK-293 cells, transfected or not with Cy5-labeled siRNAs, were analyzed in a flow cytometer using conditions for Cy5 detection (Fig. 2). Size and shape of the cells were also measured to exclude dead cells from the study. The univariate analysis of Cy5 fluorescence intensity by flow cytometry showed that the cells transfected with GAPDH Cy5-labeled siRNA have a significant amount of cell fluorescence clearly above the nontransfected cells (compare Figs. 2A and 2B). Therefore, it appears that in these experiments, the flow cytometer is able to identify those cells that contain the Cy5-labeled siRNA. This is in good agreement with previous results which have shown that, in contrast to other fluorescein-labels, siRNAs labeled with Cy3 or Cy5 could be detected by flow cytometry (20). Note that, in agreement with others (21), the efficiency of transfection of HEK-293 cells was high (Fig. 1), and only a few cells were not labeled.

To determine whether the cytometer is able to discriminate between transfected and nontransfected cells within the same cell preparation, samples of both populations were mixed. As shown in Figure 2C, the cytometer could easily identify each type of cells from the mixture, except for a middle region of about 20% of cells in which it is difficult to determine where the negative cells end and the positive cells begin.

Then, we applied the same procedure to fibroblasts, which are known to be hard-to-transfer cells, but using GAPDH siRNAs labeled with FAM (a fluorescein derivative,

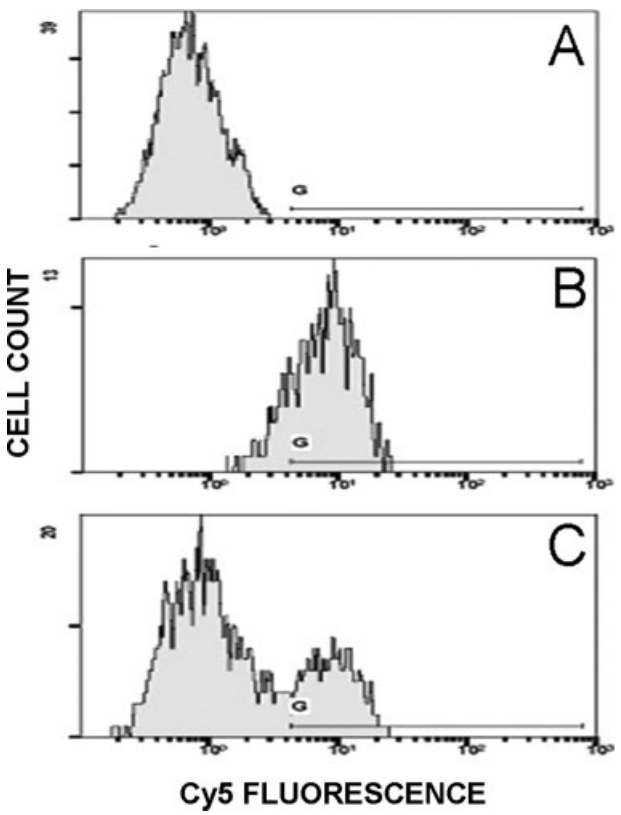

Figure 2. Detection of cells transfected with siRNA by flow cytometry. Representative histograms of Cy5-fluorescence. (**A**) Non transfected cells. (**B**) Cells transfected with GAPDH siRNA labeled with Cy5. (**C**) Mixed population of nontransfected and transfected cells (75/25%).

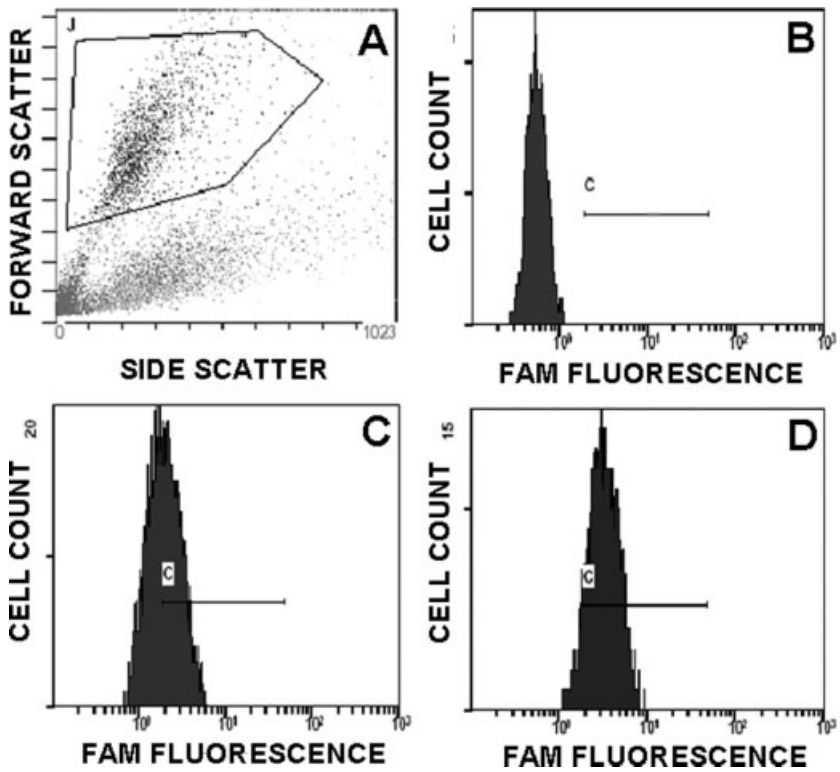

Figure 3. Different transfection efficiencies correlate with different fluorescence intensities. Human fibroblasts were transfected with FAM labeled GAPDH siRNA using different amounts of the transfection reagent. The parametric dotplot of forward and side scatter represents cell size and granularity, respectively, gate J indicating the cell population selected for fluorescence measurements (**A**). FAM positive cells were detected as positive for green fluorescence (FL1, 525 ± 20 nm). Nontransfected cells (**B**) and cells transfected with 5 (**C**) and 10 μl (**D**) of transfection reagent, respectively.

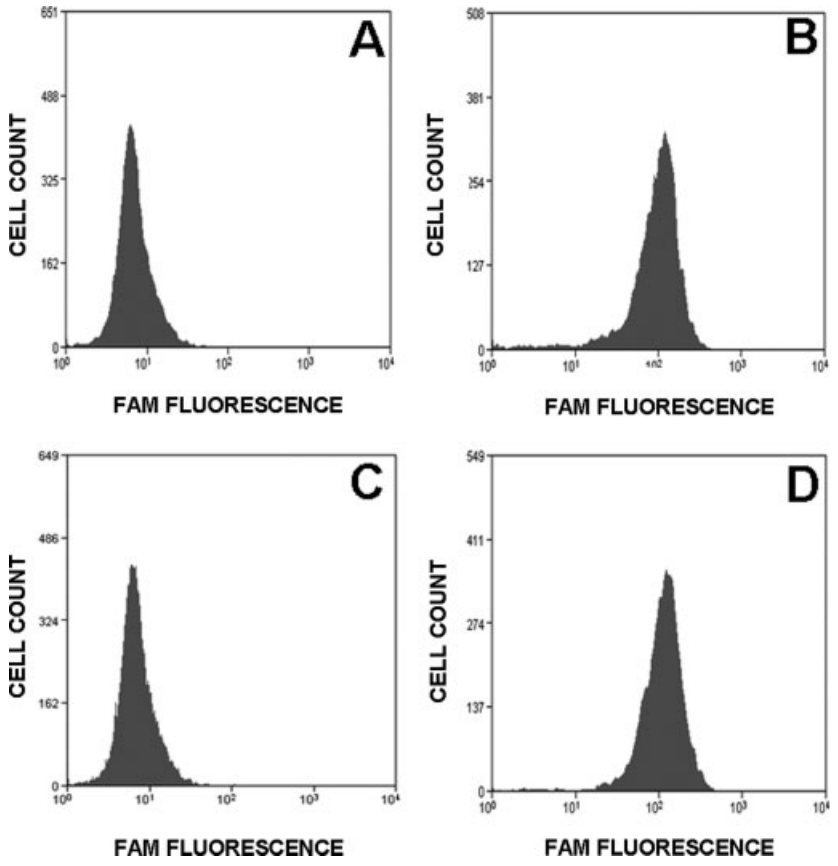

Figure 4. Further trypsinization of cells does not alter cell fluorescence. Human fibroblasts were transfected or not with FAM labeled GAPDH siRNA and detached using trypsin-EDTA as described in Materials and methods. Part of the cells were further incubated with trypsin (final concentration: 0.4 mg/ml) for 10 min at 37°C, followed by addition of soybean trypsin inhibitor (final concentration: 4 mg/ml). Then, trypsin incubated and non-incubated cells were subjected to flow cytometry. Nontransfected (**A**) and transfected (**B**) cells with no further trypsinization, and nontransfected (**C**) and transfected (**D**) cells with further trypsinization.

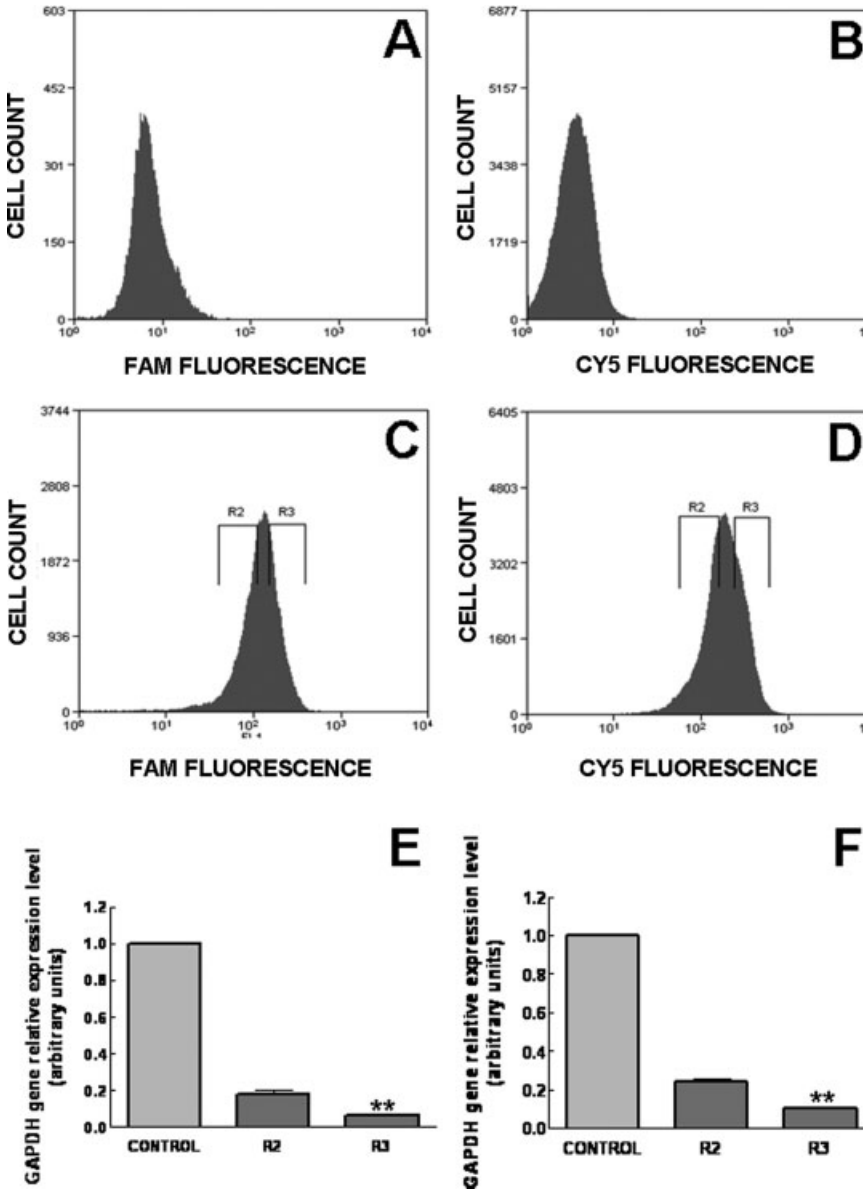

Figure 5. Separation by cell-sorting of cell populations with different silencing efficiencies. Human fibroblasts were nontransfected (**A** and **B**) or transfected with GAPDH siRNA labeled with FAM (**C** and **E**) or Cy5 (**D** and **F**). Two different populations of the transfected cells were separated in the cell sorter by their fluorescence intensity (C and D), corresponding to 40% of the cells with the highest fluorescence and 40% of the cells with the lowest fluorescence (R3 and R2, respectively) and analyzed by quantitative one step RT-PCR for the level of GAPDH mRNA (E and F). Results in E and F are the mean and SD from three separate experiments with duplicated measurements. Stars indicate differences from R2 values which were found to be statistically significant at $^{**}P < 0.005$.

22). When cells transfected with 5 and 10 μl of siPortAmine were analyzed in a flow cytometer, only a single broad fluorescence distribution was observed with a fluorescence intensity higher than in nontransfected cells (Fig. 3). This was a surprising result since we expected to find two peaks, each one corresponding to transfected and nontransfected cells. The possibility that FAM was present on the cell surface was excluded using a conventional control based on further trypsinization of the cells (Fig. 4). It appears therefore that most cells have internalized some amount of siRNA, and that the flow cytometer even detects cells containing a low number of labeled siRNA molecules. Note that although both amounts of the transfection agent produced a single fluorescence peak, fluorescence was greater at the highest amount of siPortAmine (compare Figs. 3C and 3D). Quantitative RT-PCR indicated that the reduction of GAPDH gene expression in cells treated with 5 and 10 μl of siPortAmine was (45 ± 6)% and (81 ± 5)% (mean ± SD, $n = 3$). Altogether these results indicate that the intensity of fluorescence inversely correlates with the amount of the target mRNA and that a high fluorescence intensity corresponds to a better knockdown efficiency.

Since a major goal of our work was to develop a procedure to physically separate silenced from nonsilenced cells, we decided to use a cell-sorter to distribute the population of fibroblasts transfected with FAM-labeled GAPDH siRNA into two groups of cells: those corresponding to the 40% most fluorescent cells and those corresponding to the 40% least fluorescent cells, while the remainder 20% cells, in which the fluorescence was intermediate between both populations, were rejected. When the amount of GAPDH mRNA from each group of cells was quantified by real time RT-PCR analysis, it was found that the most fluorescent population contained

about 3-fold less GAPDH mRNA than the less fluorescent population (R2) (Figs. 5C and 5E). Similar results were obtained using Cy5-labeled GAPDH siRNA (Figs. 5D and 5F).

In conclusion, in cells transfected with fluorescently labeled siRNAs, it is possible, using a cell sorter, to select the most silenced cells, which can be employed for further analysis. This procedure may be particularly useful with hard-to-transfer cells such as primary cultured cells as well as Jurkat (human acute T-cells), K562 (human erythroleukemia cells), PC12 (rat pheochromocytoma cells), and other cells.

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