Chapter 10

Applications of Aptamers in Flow and Imaging Cytometry

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Abstract

Aptamers compete with antibodies in many applications, in which high-affinity and specificity ligands are needed. In this regard, fluorescence-tagged aptamers have gained applications in flow and imaging cytometry for detecting cells expressing distinct antigens. Here we present prospective methods, as a starting point, for using these high-affinity ligands for cytometry applications.

Key words Aptamers, Fluorescence-labeling, Flow cytometry, Imaging cytometry, Automation

1 Introduction

Aptamers comprise a class of DNA or RNA molecules that specifically bind to their targets, rendering multiple applications. Due to their high specificity, aptamers are used as recognition molecules for virtually any target molecule class—ranging from small molecules (e.g. ampicillin or dopamine [1]) to proteins localized on a cell- or virus surface, respectively [2], or intracellularly in the cytoplasma or nucleus [3] as well as entire cell surfaces or organisms [4]. Aptamers with high-affinity have promising research, industrial, diagnostic and clinical applications. Although primary applications of aptamers aim at inhibition of target protein function [5], fluorescently labeled aptamers are more often used as tool for the identification and quantification of expression levels in western blotting, quantum dots [6], biosensors [7], imaging in fluorescence microscopy [2] and flow cytometry (Table 1).

The latter approach offers the advantage of obtaining several parameters measured simultaneously (3–16 parameters, including side scatter and forward scatter) in a cell population maintained in suspension. Furthermore, measurement precision is high in a short period of time, making flow cytometry the method of choice for experiments involving enumeration of mixed cell populations. In addition, aptamers have been developed for imaging purposes.

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Table 1
Example of applications for fluorescence-labeled aptamers

Related structure	Application	Target gene	Target cell type	Fluorescent probe	References
Cell membrane	Flow cytometry and fluorescence microscopy	CD44	Cancer stem cell lines	FITC	[2]
Cell membrane	Flow cytometry and fluorescent microscopy	CD8	Lymphocytes	Cy5	[8]
Cell membrane	Flow cytometry	c-kit	BJAB lymphoblastoma cell line	PE and Alexa 647	[9]
Nuclear	Flow cytometry and fluorescent microscopy	Nucleolin	HeLa	PPIX	[3]
Cytosol	Flow cytometry	Intracellular interferon-γ	Lymphocytes	FITC	[10]
Whole bacteria	Flow cytometry	Several	*Staphylococcus aureus*	5′ABFL	[11]

FITC fluorescein isothiocyanate, *Cy5* cyanine 5, *PE* phycoerythrin, *PPIX* protoporphyrin IX, *5′ ABFL* 5′azobenzylflavin

Several features of aptamers, such as their small size, conformational flexibility and easiness of modifications, turn aptamers into excellent tools for flow cytometry and cellular imaging. Aptamers can either be covalently bound to fluorophores or nanoparticles with optical properties or non-covalently labeled by using biotin–streptavidin chemistry.

More recently, with the advancement of image processing and artificial intelligence techniques, the nominated "imaging cytometry" experiment has been developed. It consists of an automatic machine to capture high-resolution images coupled to a computer with a high-end image processing software. It captures almost the same parameters as flow cytometer with the addition of two- or three-dimensional images. Image segmentation permits subcellular identification and morphology measurements (nucleus quantities and size, cytosol area, cell perimeter, and several others). This is particularly interesting for confirm the subcellular localization of a particular target. The experiment can be performed with cells attached to a surface (rich in details) or in suspension (quicker). The experiment can be with alive (for continuous monitoring) or fixed cells (easier). With live cells, it offers the advantage of time-lapse for migration tracking experiments or intracellular changes detection of particular protein expression patterns without disrupting cell microenvironment. When information in high throughput shall be acquired, it is named

"high content analysis" or "high content screening" as used for the InCell® equipment (GE LifeSciences), while "histo-cytometry" refers to this experiment in cells in an in situ context [12]. Some equipments only work with cells in suspension (Tali® Image-Based Cytometer, Life Technologies). For instance, image cytometry has been used for imaging of Ets1 expression by metastatic cells using Texas Red-labeled RNA aptamers[13].

The coupling of both aptamers and cytometry techniques produce several outcomes such as: (1) Population analysis to define the percentage of cells stained by fluorescently labeled aptamers. (2) Characterization of aptamer–target cell affinity by determination of the apparent dissociation constant (K_d of aptamers). (3) Identification of the localization of the target for a specific aptamerby imaging cytometry; (4) Determination of the cellular subtype, to which the aptamer binds by, i.e., using antibodies specific for Clusters of Differentiations (CD).

Since the experimental conditions for the use of aptamers in flow cytometry and regular fluorescence imaging experiments are essentially the same, we can conclude that both techniques work with the same methodology. Thus, here we offer a prospective method for flow cytometry that might work for imaging cytometry experiments. Bottlenecks are discussed together with troubleshooting notes.

2 Materials

2.1 Reagents (See Note 1)

1. DPBS (Dulbecco's phosphate buffer saline): 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride.
2. 2 mM EDTA solution diluted in DPBS.
3. Cell culture medium (according to the culture or tissue to be analyzed by aptamers).
4. Fixative solution (2×) for intracellular staining: DPBS plus specific fixative agents depending on tissue and cellular type (*see* **Note 2**).
5. Permeabilization Buffer (for intracellular staining): 0.1 % Triton X-100 in DPBS.
6. 5× Binding buffer: 25 mM HEPES, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 145 mM NaCl, 0.05 mg/ml yeast t-RNA, pH 7.4 (*see* **Note 3**).
7. 4.5 % (w/v) glucose solution in 1× binding buffer (*see* **Note 3**).
8. 5 % BSA solution in 1× binding buffer (*see* **Note 3**).
9. Fluorescent-labeled aptamer (*see* **Note 4**).

2.2 Others

1. 50 μm cell strainer.
2. Thermoblock.
3. Flow cytometer.
4. Centrifuge.

3 Methods

3.1 Preparation of Aptamers for Cell Labeling

1. Prepare a solution of 1.6 μM aptamers (*see* **Note 5**) in 1× binding buffer.
   (a) Example: 200 pmols of aptamer (having a 18.6 pmol/μl stock solution, we would use 10.8 μl), 40 μl of 5× binding buffer, 69.2 μl of H_2O, to a total volume of 120 μl.
2. Incubate for 10 min at 95 °C.
3. Immediately place the tube on ice for 10 min.
4. Add glucose and BSA solution to 1× at the final solution.
   (a) Example: add 40 μl of glucose and 40 μl of BSA solutions, reaching a 200 μl solution of 1 μM of aptamers in a 1× binding buffer with 0.9 % of glucose and 1 % BSA (*see* **Note 6**).
5. Incubate aptamers for 20 min at room temperature.

3.2 Preparation of Living Cells for Labeling by Aptamers

1. Remove medium. Rinse plate once with DPBS. Withdraw DPBS.
2. Add 5 ml of 2 mM EDTA solution to plate (*see* **Note 7**).
3. Mix gently and incubate at 37 °C.
4. After 5 min of incubation, mix cells gently and check for non attached cells; continue until majority of them is detached.
5. Add approximately 5 ml of culture media containing serum to dilute EDTA.
6. Remove the medium with cells and centrifuge for 5 min at 300 × *g*.
7. Remove supernatant and resuspend pellet in culture medium containing serum.
8. Gently agitate the solution for 30 min (*see* **Note 8**).
9. Centrifuge the cells, remove the medium, and resuspend them in binding buffer.
10. Pass cell suspension through a 50 μm cell strainer to eliminate cell aggregates.
11. Count the cells and separate at least 2×10^5 cells/tube.
12. Centrifuge for 5 min at 300 × *g* for pellet formation and discard the supernatant.
13. Follow aptamer incubation procedure.

3.3 Preparation of Fixed and Permeabilized Cells

Fixation and permeabilization are required for the detection of intracellular targets. However, these procedures may increase background fluorescence and change light scatter profiles. Therefore, the same procedure must be followed for all samples, which will be compared including control samples.

1. Prepare the cells as described above for living cells. Add sufficient volume of fixative into the tube containing the cells to obtain a 1× solution.
2. Incubate at room temperature for 10 min.
3. Centrifuge for 5 min at 300 × *g* and discard the supernatant.
4. Wash the pellet twice using 1× binding buffer + 1 % BSA.
5. Resuspend pellet in 500 μl of permeabilization buffer at room temperature and incubate for 15 min.
6. Centrifuge for 5 min at 300 × *g* and discard the supernatant.
7. Wash the pellet twice using 1× binding buffer + 1 % BSA.
8. Centrifuge for 5 min at 300 × *g* and discard the supernatant.
9. Follow aptamerincubation procedure and correspondent washing steps.

3.4 Incubation of Cells with Aptamers

1. In the tube, gently mix together the cell pellet (2×10^5 cells/tube) and aptamer solution (1 μM final concentration to a final volume of 200 μl).
2. Incubate mixture at room temperature gently shaking for 30 min.
3. Centrifuge for 5 min at 300 × *g* and remove the supernatant.
4. Add 200 μl of binding buffer to cells.
5. Centrifuge for 5 min at 300 × *g* and remove the supernatant.
6. Resuspend pellet in 500 μl binding buffer.
7. Use this sample for flow cytometry analysis.

3.5 Cytometry Controls

Controls must ensure to comprise:

1. Control 1: cells without any aptamer or antibody for autofluorescence determination.
2. Control 2 (or more): cells marked with only aptamers or any other marker of interest (antibodies for example) for channel leak, proper compensations and gating prior to experiments (*see* **Note 9**).

3.6 Determination of the Dissociation Constant (K_d) of Aptamer–Cell Binding with Flow Cytometry

The assessments for the target antigen-binding equilibrium dissociation constant (K_d) of aptamers in cytometry must be performed with single aptamers rather than with populations or pools of different molecules. Therefore, prior to cytometry assays, sequencing, clustering, and structural analysis of the selected molecules through the SELEX technique must be already performed.

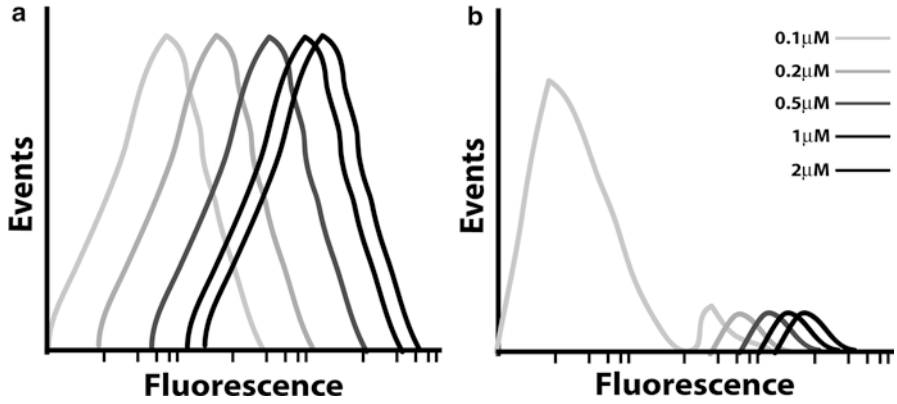

Fig. 1 Profile of fluorescence gain according to increment of aptamer concentration. (**a**) Increase of fluorescence for a population completely marked by fluorescence-tagged aptamers. (**b**) Increase of fluorescence for a subpopulation marked by a fluorescence-tagged aptamers

1. In order to assess the K_d of aptamer–cell binding, the mean fluorescence intensity of the stained population labeled by several concentrations of target aptamer is determined [9]. The equation

$$\Upsilon = B_{max} \cdot X / (K_d + X) \quad (1)$$

is used to calculate the K_d of aptamer binding, where B_{max} is the maximum percentage of fluorescence, Υ is the mean percentage fluorescence, and X is the molar concentration of the aptamer.

2. When the K_d of aptamer binding to the entire population shall be determined, the mean fluorescence intensity of the population must be considered (Fig. 1a). For measuring K_d values of aptamers that bind just to cells within a subpopulation, these need to be gated for proper quantification (Fig. 1b).

3.7 Analysis of Aptamer-Target Cell Binding by Imaging Cytometry

1. Preparation of samples for imaging cytometry follows the same rationale described for flow cytometry. The difference for imaging cytometry is that the samples instead in solution analyzed in the flow cytometer, while they are plated on slides for experiments with an imaging cytometer.
2. Both cytometry techniques are complementary, whereas one has a power of measuring larger cell populations (flow cytometry), while imaging cytometry conjugates the visual analysis for better tracking of aptamer localizations with antigen-immunostaining by antibodies.

3.8 Characterization of the Cellular Subtype Recognized by Aptamers Using Antibodies Directed against Clusters of Differentiations (CD)

Both cytometry techniques (flow and imaging cytometry) can be combined for better understanding, to which cell type fluorescence-labelled aptamers bind. Specific surface antigens are expressed depending on the cell type.

By using flow cytometry co-expression of aptamer targets and CD antigens can be verified, while imaging cytometry reveals further details on the morphology of the cells. Moreover, imaging cytometry possesses advantages over flow cytometry, when adherent cells shall be analyzed. The dissociation process using trypsin or EDTA damages cell surfaces and may affect cell viability and antigen expression patterns.

4 Notes

1. Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all solutions at room temperature (unless indicated otherwise) and all reagents for molecular biology at 4 °C. Diligently follow all waste disposal regulations when disposing waste materials.
2. Several fixative agents can be used, such as paraformaldehyde, methanol and acetone, ranging from 1 to 4 % depending on the tissue or cell type. We suggest prior standardization to optimize the fixation process for each cell type to be analyzed by flow or image cytometry.
3. This buffer should be filtered and stored at 4 °C
4. The selection of the most adequate fluorochrome combination depends on the specific optical configuration of the flow cytometer.
5. The final concentration of aptamers used for cytometry depends on the aptamer affinity for the target and experimental design according to the hypothesis to be tested.
6. Do not add the glucose or BSA prior to heat denaturation, as they can make the solution too viscous.
7. Trypsin solution can compromise cell surface targets and alter immunostaining results. For cells with highly expressed target antigens, trypsin is a valid option to speed up the removal of cells.
8. This step allows the cells to assume a more homogeneous shape, facilitating gating for side and forward scatter parameters in flow cytometry.
9. We also recommend the use of the Viability Dyes to help eliminating dead cells during cytometry analysis.

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