Mapping Receptor Density on Live Cells by Using Fluorescence Correlation Spectroscopy

Yan Chen[a],[b], Dr. Alina C. Munteanu[a],[b], Dr. Yu-Fen Huang[a], Dr. Joseph Phillips[a], Zhi Zhu[a], Michael Mavros[a], and Prof. Weihong Tan*[a]

[a]Department of Chemistry and Department of Physiology and Functional Genomics, Shands Cancer Center and Center for Research at Bio/nano Interface, UF Genetics Institute and McKnight Brain Institute, University of Florida, Gainesville, FL 32611 (USA)

Abstract

Study of the density, spatial distribution, and molecular interactions of receptors on the cell membrane provides the knowledge required to understand cellular behavior and biological functions, as well as to discover, design, and screen novel therapeutic agents. However, the mapping of receptor distribution and the monitoring of ligand–receptor interactions on live cells in a spatially and temporally ordered manner are challenging tasks. In this paper, we apply fluorescence correlation spectroscopy (FCS) to map receptor densities on live cell membranes by introducing fluorescently marked aptamer molecules, which specifically bind to certain cell-surface receptors. The femtoliter-sized (0.4 fL) observation volume created by FCS allows fluorescent-aptamer detection down to 2 molecules and appears to be an ideal and highly sensitive biophysical tool for studying molecular interactions on live cells. Fluorophore-labeled aptamers were chosen for receptor recognition because of their high binding affinity and specificity. Aptamer sgc8, generated for specific cell recognition by a process called cell systematic evolution of ligands by exponential enrichment, was determined by FCS to have a binding affinity in the picomolar range (dissociation constant $K_d = 790 \pm 150$ pM) with its target membrane receptor, human protein tyrosine kinase-7 (PTK7), a potential cancer biomarker. We then constructed a cellular model and applied this aptamer–receptor interaction to estimate receptor densities and distributions on the cell surface. Specifically, different expression levels of PTK7 were studied by using human leukemia CCRF-CEM cells ($1300\pm190$ receptors $\mu$m$^2$) and HeLa cervical cancer cells ($550\pm90$ receptors $\mu$m$^2$). Competition studies with excess nonlabeled aptamers and proteinase treatment studies proved the validity of the density-estimation approach. With its intrinsic advantages of direct measurement, high sensitivity, fast analysis, and single-cell measurement, this FCS density-estimation approach holds potential for future applications in molecular-interaction studies and density estimations for subcellular structures and membrane receptors.
Keywords
aptamers; fluorescence spectroscopy; ligand–receptor interactions; receptors

Introduction
Biological membranes are the sites where different elements of the cellular machinery are brought together; therefore, they are central to the very phenomenon of life.\textsuperscript{[1]} Significantly different from bulk water, but integrated with it, biological membranes create an environment in which many complex enzymatic reactions and bioelectrical and biochemical signaling processes occur. Examples include the conversion of metabolic energies into osmotic, electrical, and mechanical work, the transportation of materials between cellular compartments, and the processing of information. In a broad sense, many cellular activities involve membrane-based ligand–receptor interactions,\textsuperscript{[2]} which are mediated by membrane-associated proteins that are incorporated into the structures of the lipid bilayers.

Consequently, biological membranes are the primary target receptors for many drugs representing different therapeutic categories. Therefore, although knowledge of the molecular mechanisms underlying ligand–receptor interactions has theoretical significance, there are also practical implications for the discovery, design, and screening of novel therapeutic agents. The binding of extracellular ligands to receptors also allows living cells to constantly monitor and respond to changes in their environment. Therefore, the control of receptor distribution and trafficking in a spatially and temporally ordered manner is required to modulate cell behaviors that range from cell division to differentiation. Furthermore, the ability to obtain quantitative information about ligand–receptor interactions and receptor distribution over the cell surface will be of significance in our understanding of membrane-receptor characteristics, expression levels, and spatial distribution, as well as clustering and molecular changes, on the molecular level in living biological specimens. These data would also, in turn, provide an important database for drug discovery.

However, so far, only a limited number of approaches have been established for estimating membrane-receptor densities. Liquid scintillation counting, a standard laboratory method in life science, measures radiation from beta-emitting nuclides and, thus, requires the use of radioactively labeled ligands.\textsuperscript{[3]} Fluorescence subtraction, the most conventional fluorescence approach for density estimation, involves several washing steps to remove unbound ligands, and the receptor density is determined by measurement of the fluorescence from the supernatant containing the free ligands. The concentration of free ligands is estimated by interpolation from a standard linear calibration curve.\textsuperscript{[4]} However, the half-life of the receptor–ligand complex is often shorter than, or equal to, the time required for the separation of free and bound ligands. Specific interactions between certain ligands (for example, peptides, hormones, and natural products) and their different receptor subtypes are, therefore, often overlooked by the conventional fluorescence-subtraction method. In addition, the analysis may also be compromised by high background levels of other membrane proteins that are expressed endogenously on the membrane. In certain cases, receptor numbers per cell are few; therefore, no specific binding is detected because of high background levels. Alternatively, the receptor of interest could be separated and then
purified from over-expressing cells or tissue. This biochemical purification, however, typically requires exchange of the physiological lipid/lipid–protein environment by a detergent micelle, which may modify the binding properties of the receptor. In an ideal preparation format, a high enrichment of the receptor of interest would be combined with the receptor as it is found, that is, naturally integrated within its own lipid/lipid–protein environment.

To address the above problems, we report here an effective approach for direct measurement of membrane-receptor density in its natural physiological environment on the cell surface by using a highly sensitive technique, fluorescence correlation spectroscopy (FCS). FCS is an ideal biophysical and bioanalytical tool for studying concentrations, propagation, interactions, and internal dynamics of molecules at nanomolar concentrations in living cells.\(^5,6\) It is capable of analyzing even minute fluorescence-intensity fluctuations caused by the equilibrium of a small ensemble of molecules. These specific fluctuations in frequency act like the “fingerprint” of a molecular species that is detected when it enters and leaves the instrument’s femtoliter-sized, optically defined observation volume, which is created by a focused laser beam. This small laser volume element (<1 fL), about 250 nm in diameter, allows the detection of single molecules.\(^7\) as well as the measurement of molecular properties at specific coordinates on the cell membrane or inside cells.\(^8\) However, although this highly sensitive technique has been developed and adapted to cellular measurements ever since the breakthrough in the early 1990s, there has, so far, been no report on its application for receptor-density studies on live cell membranes. With its single-molecule detection sensitivity, FCS allows the detection of receptor binding sites at the molecular level in a cell membrane’s native environment on the cell surface.\(^8,9\) Thus, it permits the identification of target receptors, as well as the measurement of detailed ligand–receptor interaction kinetics. By measuring ligand–receptor interactions on individual cells, FCS can be used to obtain binding affinity and receptor-density information from just a small number of cells (less than 50), which was not possible before by conventional binding techniques, such as flow cytometry. This makes our approach critical in certain situations in which it is not feasible to obtain millions of cells. Also, the practicality of the FCS technique arises from the elimination of the washing steps that are otherwise needed to separate unbound from bound ligands.\(^7\) The changes in diffusion times for ligands upon binding to membrane receptors enable FCS to differentiate between the receptor’s bound and free ligand fractions.

In order to mark the membrane receptors of interest for the density studies with FCS, fluorophore-labeled nucleic acid aptamers were chosen for receptor recognition. As probes for molecular recognition, the efficacy of DNA aptamers has been successfully demonstrated in many applications.\(^10,11\) Aptamers are single-strand oligonucleotides derived from a selection process called systematic evolution of ligands by exponential enrichment (SELEX). In our study, the aptamers were selected from whole intact biological live cells through a process called cell-SELEX,\(^11\) and these aptamers are capable of binding to their target molecules on the cell-membrane surface with high affinity and specificity.\(^12\) In comparison with the molecular probes currently available for receptor recognition, such as monoclonal antibodies, aptamers offer significant advantages over existing antibody-based recognition procedures in that they offer higher binding affinity (higher retention/reduced
dissociation) and specificity to the target (ability to determine variations on the protein target down to single amino acid changes), higher selectivity against mutated protein epitopes, and potentially reduced immunogenicity and increased tumor penetration associated with their size. They also possess lower molecular weights and can be chemically synthesized and easily modified.\textsuperscript{[10,11a]} These features make aptamers promising probes for recognizing target-specific receptors and identifying binding sites on the cell-membrane surface, sites that could also be recognized as potential bio-markers.\textsuperscript{[13]} Nowadays, more and more aptamers have been generated as specific probes for molecular signatures on the cell surface. However, there are only limited ways to study them fully and use them for biological studies. Herein, we combine aptamer recognition with the highly sensitive FCS tool to perform a ligand–receptor interaction study on the cell membrane. This technique yields the information required to estimate receptor density and distribution and, thus, extends the potential applications of aptamers generated from cell-SELEX, as well as proving FCS to be an effective biophysical tool for study of the cell-membrane surface.

By using an in vitro cell-SELEX procedure,\textsuperscript{[11]} we selected an aptamer, sgc8, towards the T-cell ALL CCRF-CEM cell line. We also successfully elucidated the target protein, human protein tyrosine kinase-7 (PTK7), by using this newly selected aptamer.\textsuperscript{[14]} PTK7 has been discovered to be highly expressed on the cell membrane in a series of leukemia cell lines.\textsuperscript{[13]} It is recognized as a potential cancer bio-marker, with a role characteristic of tumors, that is, a signal amplifier or modulator.\textsuperscript{[15]} In order to demonstrate FCS as an effective approach for mapping receptor densities on live cells, we chose two different cell types with different expression levels of PTK7 on the cell membrane for a proof of principle. This is the first study that uses FCS to estimate the density of the membrane receptor PTK7 on different cell types through aptamer–receptor interactions.

### Results and Discussion

#### FCS measurement and analysis

Fluorescence correlation spectroscopy measurement is accomplished by focusing an excitation laser beam onto the sample and then monitoring the fluorescence fluctuations derived within the focal region of the laser beam (Figure 1). Diffusion of fluorophores into and out of the focal volume alters the local concentration of fluorophores and, thus, gives rise to spontaneous fluorescence-intensity fluctuations. Correlation of the fluorescence-intensity fluctuations at time $t$, $\delta I(t)$, with those at a later time $(t+\tau)$, $\delta I(t+\tau)$, over the mean fluorescence intensity, $<I>$, yields the normalized intensity autocorrelation function, $G(\tau)$ [Eq. (1)].

\[
G(\tau) = 1 + \frac{<\delta I(t)\delta I(t+\tau)>}{<I>^2}
\]  

(1)

We are then able to monitor the real-time aptamer–receptor interactions on the cell membrane by tracking the diffusion of the fluorescently labeled aptamers into and out of the focal volume. More specifically, a derivative of $G(\tau)$, which assumes a single-component
solution and only considers diffusion along the 3D axial dimensions, $\omega_{xy}$ and $\omega_z$, of the laser beam, yields Equation (2).

$$G_{3D}(\tau) = \frac{1}{N} \frac{1}{1 + \frac{\tau}{\tau_D}} \frac{1}{\sqrt{1 + \left(\frac{\omega_{xy}}{\omega_z}\right)^2 \tau_D}}$$ (2)

The amplitude of $G(\tau)$ depends on the absolute number of molecules, $N$, occupying the observation volume. A higher number of molecules in the observation volume (higher concentration) results in a lower correlation amplitude. The mean diffusion time, $\tau_D$, describes the average time it takes for a molecule to diffuse through the observation volume. It works as a characteristic fingerprint for specific molecules in specific diffusion states, because molecules with different molecular weights take different times to diffuse through the same observation volume and the same molecule also diffuses differently in different states, for example, free in solution or bound to the membrane surface. The dimensions of the observation-volume element are defined by the half-axes in length ($\omega_z$) and width ($\omega_{xy}$), as shown in the enlarged diagram in Figure 1. A 0.4 fL ellipsoid-shaped confocal volume with half-axes of $\omega_{xy} = 0.22 \mu m$ and $\omega_z = 1.56 \mu m$ was obtained from free-dye calibration of Rhodamine 123, a photostable dye with a known diffusion coefficient ($D = 3 \times 10^{-10} \text{m}^2\text{s}$).

This femtoliter-sized, optically defined observation volume is the engineering feature that makes FCS a highly sensitive biophysical tool for studying molecular interactions on cell membranes.

**Binding of aptamers to membrane receptors**

For the aptamer–receptor binding study, light coming from a laser beam is focused on the cell membrane. If we consider that the thickness of the lipid bilayer ($\approx 4 \text{nm}$) is 3 orders of magnitude smaller than the typical axial length ($\omega_z = 1.56 \mu m$) of the FCS confocal volume in its geometry, for the fluorophore-labeled aptamer binding to a cell membrane, the diffusion at the membrane takes place only at a 2D surface, which will be the surface section that is equal to the confocal volume. The diffusion of bound aptamers on the membrane can then be treated as two-dimensional diffusion, and $G_{2D}(\tau)$ is represented by Equation (3).

$$G_{2D}(\tau) = \frac{1}{N} \frac{1}{1 + \frac{\tau}{\tau_D}}$$ (3)

Equations (2) and (3) represent the time-dependent correlation function for 3D and 2D diffusion of aptamers, respectively. When the volume element with half-axes $\omega_{xy}$ and $\omega_z$ is projected onto the cell surface, not only bound aptamer diffusing at the cell surface, but also free aptamer diffusing above the cell surface, will be seen. When some of the aptamers in the confocal volume bind to the membrane receptors, the bound aptamers are restricted to the cell surface and diffuse more slowly. Thus, the diffusion time of the bound aptamers increases compared to that of the free ones. Diffusions of free aptamers above the cell...
surface are described by the 3D diffusion function, whereas diffusions of bound aptamers on the cell membrane are represented by the 2D diffusion function. Thus, if the aptamer–receptor complex is stable for the timescale of its transit through the detection region, then the overall autocorrelation function describing the activities that occur in the confocal volume will represent a linear combination of the autocorrelation functions of free and bound aptamers, as described by Equation (4), in which \( N \) is the total absolute number of fluorescent molecules inside the focus, \( \tau^\text{free} \) is the diffusion time for the unbound labeled aptamer, \( \tau^\text{bound} \) is the diffusion time for the bound labeled aptamer, \((1-r)\) is the fraction of unbound aptamer diffusing with \( \tau^\text{free} \), and \( r \) is the fraction of bound aptamer diffusing with \( \tau^\text{bound} \).

\[
G(\tau) = \frac{1}{N} \left( (1-r) \cdot \frac{1}{1 + \frac{\tau^\text{free}}{\tau D}} + \frac{1}{1 + \frac{\tau^\text{bound}}{\tau D}} \right)
\]

(4)

Autocorrelation functions of FITC-labeled aptamers in solution and bound to the cell membranes, respectively, are shown in Figure 2a. We see that the binding of aptamers to receptors results in an increase of the diffusion time, with \( \tau^\text{D} = 0.827 \text{ ms} \) for the bound aptamers, relative to \( \tau^\text{D} = 0.235 \text{ ms} \) for the free ones; thus, FCS is able to differentiate bound aptamers from free ones. The corresponding fluorescence-intensity fluctuations are shown at the bottom of Figure 2a. Stable fluorescence fluctuations show that no photo-bleaching effects occurred during the entire detection time in the detection volume. Control experiments with a randomized sequence of 41 nucleotides (Library, Figure 2b), which has been shown as the negative binding control to receptor PTK7 in cell-SELEX,\(^{11a}\) show similar diffusion times for aptamers that are free in solution and those on the cell membrane. This result indicates that only specific interactions between aptamers and membrane receptors will give the response of increased diffusion time on the cell surface.

**Specificity of binding by the aptamers**

The specificity of aptamer–receptor interactions, as demonstrated by the specific labeling of target receptors by aptamers, provides the basis for studying receptor density by using FCS. The results shown in Figure 3 demonstrate the specific binding of aptamer sgc8 at different concentrations with its membrane-bound receptors in different cell types. The formation of complexes between the receptors and aptamers is identified by the change in the autocorrelation function in the positive target cells, which is not shown in the negative control cells that lack PTK7 expression on the cell membrane.\(^{11a,17}\)

**Binding affinity**

Study of the density and distribution of membrane receptors at the molecular level in living biological specimens has been challenging. The above experiments have shown the efficacy of FCS in terms of ligand-binding specificity to membrane receptors. However, because FCS
is also capable of measuring detailed molecular interactions between ligands and receptors, this property was applied to study the binding affinities of aptamer–receptor interactions in their native physiological environment on the cell membranes. The resulting data, in turn, provide us with the aptamer concentration required to saturate all of the receptor binding sites in the density study. For this purpose, we carried out binding experiments on individual cells with different concentrations of aptamers. While keeping the same amount of HeLa cells in different wells in the culture dish, we varied the concentration of FITC-labeled sgc8 aptamers from 0.1 to 3.0 nM. We focused on the cell membrane with the 0.4 fL ellipsoidal-shaped confocal volume from a 1.2 mW laser beam, the geometry of which had been determined from the free-dye calibration with half-axes of $\omega_{xy} = 0.22 \, \mu m$ and $\omega_z = 1.56 \, \mu m$ (Figure 2). As a result of the difference in the diffusion times of bound versus free aptamers, we were able to assess the percentage of bound aptamers ($r$) by fitting the autocorrelation according to Equation (4). The fitting of the autocorrelation curve also yielded the absolute number of total aptamers ($N$) inside the confocal volume, which was interpreted as the reciprocal of the autocorrelation amplitude (in Equation (4), when $\tau$ approaches 0, $G(0) = 1/N$). As the total aptamer concentration increases, more and more aptamers bind to the membrane receptors, so the number of bound aptamers inside the confocal volume (total aptamer number×bound fraction, $N\times r$) correspondingly increases, until it reaches saturation. We plotted each bound aptamer number ($N\times r$) versus the corresponding total aptamer concentrations used. As can be seen in Figure 4 and Table 1, higher concentrations of FITC–sgc8 clearly lead to a larger bound aptamer number (Table 1, right-hand column). The femtoliter-sized (0.4 fL) observation volume created by FCS also allowed the detection of fluorescent aptamers down to a number of about 2, if a low concentration (0.1 nM) of aptamer was incubated with the cells; this result substantially validates FCS as a highly sensitive biophysical tool for studying molecular interactions on live cells. In the sgc8-binding curve with human cervical cancer HeLa cells, a picomolar-range dissociation constant ($K_d = 790 \pm 150 \, pM$) was determined by nonlinear regression. This $K_d$ result is in good agreement with the flow cytometry result ($K_d = 810 \, pM^{[11a]}$), which further confirms the validity of using FCS to detect and quantify aptamer–receptor interactions. The binding curve also indicates that a 3.0 nM aptamer concentration of FITC–sgc8 is sufficient for saturating the PTK7 receptor binding sites on HeLa cells in the FCS experiments. However, only a small amount of cells (less than 60 in total) was needed here to obtain the binding curve for aptamer–receptor interactions with FCS, whereas millions of cells are required to obtain a similar binding curve with flow cytometry. This makes FCS an invaluable technique for obtaining binding information under conditions from which only a limited amount of cells can be obtained.

**Density study**

The receptor density was obtained from each individual cell surface through aptamer–receptor binding. Experiments were performed at 4°C to inhibit the receptor-mediated endocytosis of aptamers$^{[17]}$ and, thereby, ensure the efficient saturation of receptor binding with aptamers on the membrane. When the volume element is projected onto a single cell surface, in the presence of excess FITC-labeled aptamers, both the bound FITC-labeled aptamer diffusing at the cell surface and the unbound FITC-labeled aptamer diffusing above the cell surface are observed. Thus, the parameter $N$, which could be obtained from the
fitting of the autocorrelation function, as discussed earlier, characterizes the total absolute number of aptamers inside the confocal volume, which is the sum of bound aptamers on the membrane and free aptamers above the cell surface. Whereas \( r \) represents the bound fraction of aptamers, \( (N \times r) \) stands for the total number of bound aptamers. It is assumed that the waist of the confocal-volume ellipsoid is situated on the cell membrane such that the highest fluorescence intensity is given. In this case, the surface area on the membrane covered by the focus, in which all of the bound aptamers are located, will be a circular area with a radius equal to \( \omega_{xy} \) (shown in the enlarged section of Figure 1). Therefore, the receptor density estimated in the confocal volume can be expressed as the total number of bound aptamers divided by the area covered [Eq. (5)].

\[
\text{Density} = \frac{N \times r}{\pi (\omega_{xy})^2} \quad \text{(5)}
\]

50 HeLa cells were investigated by using aptamer sgc8 to carry out the experimental measurements and determine the PTK7-receptor density on the cell surface per unit area by applying the above formula. The 0.4 fL ellipsoid-shaped confocal volume from a 1.2 mW laser beam with half-axes of \( \omega_{xy} = 0.22 \mu m \) and \( \omega_z = 1.56 \mu m \) was projected onto each cell membrane. The circular area covered by the focus volume could be estimated to be \( \pi \times (\omega_{xy})^2 = \pi \times (0.22 \mu m)^2 = 0.15 \mu m^2 \). Within this tiny covered area, the number of specific receptors that occupied the area could be interpreted from the amount of specifically tagged aptamers under the saturation conditions. As determined from the earlier binding-affinity study, a 3.0 nM aptamer concentration of FITC–sgc8 was used to saturate all of the PTK7 binding sites on HeLa cells. Autocorrelation curves were obtained from the membranes of 50 individual cells. An average of \( N \times r = 84 \) bound sgc8 aptamers was obtained, with a variation of ±14, which indicated that an average of 84 bound aptamers occupied the 0.15 \( \mu m^2 \) covered area in the confocal volume. By applying these parameters to Equation (5), the receptor density is obtained. A Gaussian-shaped distribution of the receptor density is shown in Figure 5. A mean density of around 550 receptors \( \mu m^{-2} \) is obtained, with a variation of about 90 receptors \( \mu m^{-2} \). A key advantage of the FCS density approach is suggested by this result. That is, the method gives not only the mean density, but, because of the ease of using a single-cell approach to measure multiple cells, it also gives the statistical distribution among different cells.

In order to compare the expression levels of the same receptors on the membrane of different cell types, aptamer sgc8 is applied to label the PTK7 receptor on both human cervical cancer HeLa cells and CCRF-CEM leukemia cells. A higher receptor density (about 1300±190 receptor \( \mu m^{-2} \)) is found on CEM cells than on HeLa cells (550±90 receptor \( \mu m^{-2} \)), which indicates the higher PTK7 receptor expression level on the CEM cell surface. Alternatively, a higher receptor density per unit area also indicates that the distance between two adjacent receptors is smaller for CEM cells (≈28 nm) than for HeLa cells (≈43 nm). However, in contrast to HeLa cells, a broader distribution of density was obtained for CEM, as shown in Figure 6. With these results, this expression-level study demonstrates that the use of FCS provides a potential tool for drug-delivery studies because it offers a means of interpreting
the loading ability for drugs onto the cell surface through binding to membrane proteins. Specifically and simply, the loading factor impacts the quantity of a given drug that can be internalized by receptor-mediated endocytosis. Obviously, then, different receptor expression levels on different cell types will yield distinct loading efficiencies for the same drug into these cells.

In order to demonstrate the robustness of using FCS to measure receptor density, especially in the presence of a high background signal from free labeled ligands, we also chose a weaker binding ligand, aptamer KK1H08, to prove the capability of FCS to monitor bound ligands from high concentrations of free ligands in order to determine receptor densities on the cell membrane. KK1H08 was selected for K-562 cells and showed a low binding affinity, with $K_d = (296\pm41)$ nM.\cite{1} It requires a high concentration of total aptamers (0.5 μM) to saturate all of the aptamer-binding sites. Our FCS studies prove that it can differentiate bound aptamers from a high background of free ones. A $K_d$ value of $(271\pm29)$ nM was obtained and used to determine the receptor density on the cell membrane (see the Supporting Information, Figure S2). Further experiments with excess labeled aptamer sgc8 (250 nM) incubated with HeLa cells and tested by FCS also prove the capability of FCS to differentiate bound aptamers from a high background of free aptamers (bound fraction=16±1%). In addition, the receptor density determined under these conditions (459±29 receptor μm$^{-2}$) is comparable to the one determined previously with low aptamer concentrations (550±90 receptor μm$^{-2}$; see the Supporting Information, Figure S3); this result further demonstrates the robustness of the receptor-density determination with FCS.

**Competition studies and trypsin experiments**

In order to make certain that the method developed here is effective, control experiments were conducted to further confirm the calculated results. To verify that the receptor density is obtained through specific labeling of the receptors by using fluorophore-labeled aptamers, a competitive displacement with nonlabeled aptamer was examined (Figure 7a and b). In this experiment, cells were first incubated with 3.0 nM labeled aptamers, and after 40 min, a 1000-fold molar excess of nonlabeled aptamer was added to compete against the labeled aptamer in binding to the target receptor. The number of receptors on the surface is limited so the majority should be bound by the nonlabeled aptamer, given the huge excess of the nonlabeled population. If the labeled aptamers are displaced by unlabeled aptamers, which signifies competition between them, it would indicate that the aptamers are interacting with their target receptor specifically. From Figure 7, it can be observed that the addition of nonlabeled aptamers did result in reductions of both the fluorescence count rate (fluorescence intensity) on the cell membrane and the membrane-bound aptamer density (Figure 7a and b, respectively). After about 90 min, the aptamer binding was almost entirely displaced. This proves that the labeled aptamers recognized the target receptor specifically and did bind to them on the cell membrane. Control experiments (circles in Figure 7a and b) with a randomized sequence of 41 nucleotides, which have been shown to be the negative binding control for the PTK7 receptor in cell-SELEX,\cite{11a} were conducted under the same conditions. No significant decreases of density and count rates were observed within the first 60 min relative to the competition studies with non-labeled sgc8. The small decrease after 60 min is believed to come from the dissociation rate of the labeled sgc8 itself ($k_{off}$) as the time
increased, but not from specific-binding competition. Further experiments with proteinase trypsin treatment (Figure 7c) also indicated that, when the target protein receptors were removed from the cell surface with proteinase and their density was reduced artificially, the binding of aptamers on the membrane was decreased. This, in turn, resulted in a decreased membrane-bound aptamer density obtained from FCS detection. This evidence serves to further validate the results obtained from the above density study because the aptamer must have been binding to its target and not simply undergoing a nonspecific interaction with the cell-membrane surface. Overall, these results support the use of FCS as a comprehensive tool for detailed receptor–ligand interaction studies, such as the determination of the binding affinity ($K_d$), dissociation rate ($k_{off}$), and other kinetic parameters, which other density-study approaches cannot accomplish.

Control cell line for method evaluation

To further confirm the reliability of the approach for density estimation, experiments with a well-studied system of immunoglobulin E (IgE) receptors on the surface of the RBL-2H3 cell line were also conducted by using FCS. A dissociation constant of (0.81±0.21) nM was obtained, and a 2.5 nM IgE concentration was indicated to be sufficient for saturating the IgE receptor binding sites on the FCS (see the Supporting Information, Figure S4). 30 RBL-2H3 cells were investigated to determine the IgE receptor density on the cell surface per unit area by applying Equation (5). Similar to our sgc8-binding studies, a Gaussian-shaped distribution of receptor density was obtained (see the Supporting Information, Figure S5). A mean density of around 1200 receptor μm$^{-2}$ was obtained with a variation of about 60 receptor μm$^{-2}$. We determined the surface area of the RBL-2H3 cells from confocal images of 80 individual cells (240±20 μm$^2$ cell$^{-1}$), and the IgE receptor density could be estimated to be (2.88×10$^5$±1200) receptors per cell.

As shown in Table 2, the $K_d$ value of IgE-receptor complexes obtained by FCS gave results comparable to the fluorescence quenching method, as reported in the literature, and a density with the same order of magnitude was also obtained for the IgE receptor, which greatly supports the validity of the FCS approach for density estimation.

Conclusions

In conclusion, we have reported the use of a highly sensitive technique, fluorescence correlation spectroscopy, for mapping receptor densities on live cell membranes by introducing fluorescently marked aptamer molecules, which target specific membrane receptors with high affinity and selectivity. Full saturation of aptamer binding to the cell surface is obtained at picomolar concentrations, which indicates the high-affinity binding of the aptamer–receptor complexes ($K_d = 790 \pm 150$ pM). The binding properties of aptamer–receptor complexes were investigated and further applied for the determination of receptor densities. Human protein tyrosine kinase-7 was found to have a larger density in CCRF-CEM human leukemia cells (1300±190 receptor μm$^{-2}$) than HeLa cervical cancer cells (550±90 receptor μm$^{-2}$). Competition studies and control cell-line experiments proved the validity of the density-estimation approach. This strongly indicates the intrinsic advantages of the FCS approach over conventional methods for receptor-density studies. 1) FCS
measures receptor density in the natural cell-surface physiological environment. 2) There is no need for radioactively labeled ligands or washing steps to remove unbound ligands, which results in a fast and direct detection of receptor density. 3) Beyond its application for density estimation, FCS also provides detailed kinetics information about ligand–receptor interactions, which may not be available from other density-study approaches. 4) Single-cell information and density-distribution patterns can be obtained, which is almost impossible with other strategies. 5) Only a small number of cells are needed for FCS to perform ligand–receptor interaction studies, as well as density estimation, compared to the requirements for other methods, which makes FCS a critical tool in situations where only a limited amount of cells can be obtained. 6) Receptor-expression levels and distribution patterns on different cell types can be easily obtained from FCS detection. This provides basic information for drug-loading efficiency on cell membranes and serves as a potential tool for drug-delivery studies. 7) For receptor-density estimation, FCS has an advantage over conventional approaches in that it eliminates the need for standard-curve calibration. 8) The femtoliter-sized observation volume created by FCS permits the detection of fluorescent molecules down to two and, therefore, appears to be a highly sensitive biophysical tool on live cells, especially for studying weak molecular interactions. In summary, our FCS density-estimation approach can effectively improve detection sensitivity and may have wide applications in molecular recognition and interaction studies, as well as density estimations for particles and other membrane receptors.

**Experimental Section**

**Cell lines**

CCRF-CEM (human leukaemia), HeLa (cervix adenocarcinoma), K-562 cells (CCL-243, human chronic myelogenous leukemia), and RBL-2H3 (rat basophilic leukaemia) cell lines were obtained from the American Type Culture Collection (Manassas, VA). CCRF-CEM and HeLa cells were cultured in RPMI-1640 medium (American Type Culture Collection), with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 0.5 mgmL$^{-1}$ penicillin–streptomycin (American Type Culture Collection) at 37°C under a 5% CO$_2$ atmosphere. RBL-2H3 cells were cultured in Dulbecco’s modified Eagle’s medium (American Type Culture Collection), with 15% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C under a 5% CO$_2$ atmosphere. All cells were grown in 8-well Nunc chambers (Nalge Nunc Inc., IL) to a density of approximately 1000 cells per well. The cells were washed before and after aptamer incubation with Dulbecco’s phosphate buffer (Sigma) containing 5 mM MgCl$_2$.

**Aptamer synthesis**

Aptamer sgc8 (5′-ATC TAA CTG CTG CGC CGG GAA AAT ACT GTA CGG TTA GA-3′) and aptamer KK1H08 (5′-ATC CAG AGT GAC GCA GCA GAT CAG TCT ATC TTC TCCTGA TGG GTT CCT AGT TAT AGG TGA AGC TGG ACA CGG TGG CTG AGT-3′) were synthesized on an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). The aptamer was labeled with 5′-FITC modifier. A DNA library containing a randomized sequence of 41 nucleotides was used as a control. The completed sequences were then deprotected in ammonium hydroxide/40% aqueous methyamine (AMA; 1:1) at 65°C for 20 min and further purified with reversed-phase HPLC (ProStar;
Varian, Walnut Creek, CA) on a C-18 column. A Cary Bio-300 UV spectrometer (Varian, Walnut Creek, CA) was used to measure absorbance to quantify the manufactured sequence.

**Antibody**

FITC-labeled IgE antibody was purchased from Miltenyi Biotec Company, Auburn CA (USA).

**Trypsin treatment of cells**

Cells were first washed with washing buffer (500 mL) to remove the fetal bovine serum in the medium or the binding buffer, because this might quench the function of trypsin. The cells were then incubated with a solution of 10x diluted trypsin (500 mL, 0.005%) and ethylenediaminetetraacetate (EDTA; 0.53 mm) in Hanks’ Balanced Salt Solution (HBSS) at 37°C. The fluorescence intensity and autocorrelation of bound aptamers on a single cell membrane were monitored for 90 min by using FCS.

**FCS instrumental setup**

A light beam of 20 mW coming from an Ar+ laser is routed through 2 mirrors and expanded by a beam expander, which is made up of a plan-concave lens and a plan-convex lens (Figure 1). The intensity of the excitation light is attenuated by a neutral density filter. The expanded beam is reflected by two other mirrors and enters the back port of an inverted microscope, where the laser beam is reflected by a dichroic mirror and then focused into the sample through a microscope objective lens. The fluorescence from the sample is collected by the same microscope objective lens. The filtered fluorescence is focused by the tube lens of the microscope and exits through the side port with a focus close to the body of the microscope. The signal is then filtered with a band-pass emission filter and focused onto a multimode fiber, which works as a pinhole. The fiber is coupled to a single-photon-counting module (SPCM), which detects the fluorescence signal. The detector sends the signal to the input channel of the hardware correlator. The laser beam intensity at the objective outlet was detected to be 1.2 mW, sufficient excitation power for single-photon experiments.\(^5\)

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We acknowledge the support of National Institutes of Health and National Science Foundation grants.

**References**


Figure 1.
A schematic representation of the FCS instrumental setup and an illustration of aptamer–receptor binding events on the cell membrane inside the focus. The enlarged diagram illustrates the geometry of the confocal volume with half-axes in length ($\omega_z$) and width ($\omega_{xy}$). FITC: fluorescein isothiocyanate.
Figure 2.

a) Top: Autocorrelation functions of aptamer sgc8 (10 nM) free in solution (■) and aptamer sgc8 (10 nM) incubated with HeLa cells and bound to PTK7 membrane receptors on the cell surface (∆). The diffusion time ($\tau_D$) of aptamer sgc8 is increased from 0.235 ms (free) to 0.827 ms (bound). Bottom: Fluorescence intensity (or count rate) fluctuation curves during the detection time (30 s) for aptamer sgc8 (10 nM) during free diffusion in solution (upper trace) or bound to the membrane surface (lower trace). Stable fluorescence fluctuations show no photo-bleaching in the detection volume during the entire detection time. b) Control binding experiments with a randomized sequence of 41 nucleotides (Library) were conducted under the same conditions. Both the free library and the library incubated with HeLa cells show similar diffusion times (free: 0.435 ms; bound: 0.457 ms), which indicates
that no binding interactions occur between the library and the cell membrane receptors [10 nM free Library (black line); 10 nM Library + HeLa (gray line)]. The noisy curves represent the data collected from FCS and the smooth curves are the respective fitting curves.
Selective binding of aptamers sgc8 to positive cells (a) rather than negative controls (b). Different concentrations of aptamers were incubated with positive and negative cells for 40 min at 4°C before FCS measurements. Flow cytometry results have proved Ramos cells to be a negative control cell line for sgc8 binding because these cells lack PTK7 expression on the membrane.\textsuperscript{[11a]} For each FCS measurement, 1.2 mW laser intensity was used at the objective outlet. The sample was exposed to the laser for 30 s to obtain the autocorrelation curve. A stable fluorescence intensity was observed during the 30 s detection time, which indicates no photobleaching. For positive cells, increased concentrations of aptamers lead to a decreased autocorrelation amplitude.
Figure 4.
Binding of FITC-sgc8 to the cell membrane on human cervical HeLa cells. The number of membrane-bound labeled aptamers ($N \times r$) was obtained as a function of the total aptamer concentration in the binding buffer. HeLa cells were incubated with buffer containing different concentrations of labeled aptamers for 40 min at 4°C. Each data point represents the mean of five separate measurements. For each measurement, 1.2 mW laser intensity was used at the objective outlet. The confocal volume was determined by free-dye calibration to be 0.4 fl with half-axes of $\omega_{xy} = 0.22 \mu m$ and $\omega_z = 1.56 \mu m$. The sample was exposed to the laser for 30 s to obtain the autocorrelation curve. Stable fluorescence intensity was observed during the 30 s detection time, which indicates no photobleaching. Different numbers of bound aptamers in the confocal volume at different aptamer incubation concentrations were calculated from the fitting of autocorrelation curve and are listed in Table 1. The equilibrium dissociation constants ($K_d$) of the aptamer–cell interaction were obtained by fitting the dependence of the fluorescence intensity of specific binding on the concentration of the aptamers to the equation $Y = B_{max}X/(K_d + X)$, by using Microcal Origin 6.0 software. $X$: Total aptamer concentration added into the system. $Y$: The number of bound aptamers on the membrane obtained from FCS. $B_{max}$: the maximum specific binding to be fit.
Figure 5.
Distribution of PTK7 receptor density for HeLa cells. HeLa cells were incubated with 3.0 nM sgc8 aptamers for 40 min at 4°C to saturate all of the receptor binding sites before FCS measurement. For each FCS measurement, 1.2 mW laser intensity was used at the objective outlet. The confocal volume was determined by free-dye calibration to be 0.4 fL with half-axes of $\omega_{xy} = 0.22 \, \mu m$ and $\omega_z = 1.56 \, \mu m$. The sample was exposed to laser for 30 s to obtain the autocorrelation curve. Stable fluorescence intensity was observed during the 30 s detection time, which indicates no photobleaching. The receptor density was obtained from individual cell detection by using Equation (5). The density distribution for 50 cells is shown.
Figure 6.
Comparison of PTK7 receptor density for HeLa and CEM cells. PTK7 receptor densities were obtained from the FCS measurements on 50 individual cells for each cell line and were calculated by using Equation (5). The mean PTK7 receptor density on CEM cells was found to be about 1300 receptor $\mu m^{-2}$ with a variation of about 190 receptor $\mu m^{-2}$. HeLa cells have a mean receptor density of around 550 receptor $\mu m^{-2}$ with a variation of about 90 receptor $\mu m^{-2}$. 
Figure 7.
a) Time course of the fluorescence count rate for the displacement of FITC-labeled sgc8 aptamers by nonlabeled sgc8 aptamers on the membranes of HeLa cells (■). Cells were first incubated with 3.0 nM FITC–sgc8 for 40 min at 4°C. A 1000-fold molar excess of nonlabeled sgc8 was then added to compete against the labeled sgc8 in binding to the target receptor PKT7. Fluorescence count rates were monitored for 90 min. Competition studies were also performed with a control sequence, which is a nonlabeled DNA library containing a randomized sequence of 41 nucleotides (○). HeLa cells were first incubated with 3.0 nM of FITC-sgc8 for 40 min at 4°C. A 1000-fold molar excess of nonlabeled control sequence (Library) was then added to compete against the binding of labeled sgc8 to the target receptor PKT7. b) Time course of normalized membrane-bound aptamer density change upon displacement of FITC-labeled sgc8 aptamers by nonlabeled sgc8 aptamers on HeLa cell membranes (■). The same reaction condition as those described in (A) were used. Changes in membrane-bound FITC–sgc8 density were monitored for 90 min. Competition studies were also performed with the control sequence (○). c) Time course of normalized density change with trypsin treatment (■) on HeLa cell membranes. Cells were first
incubated with 3.0 nM FITC–sgc8 for 40 min at 4°C to saturate all of the receptor binding sites. A 10× diluted trypsin solution was then added. Changes in membrane-bound FITC–sgc8 density were monitored for 90 min at 37°C.
Table 1

Binding of labeled aptamer FITC–sgc8 to the cell membrane on human cervical HeLa cells. The number of membrane-bound labeled aptamers (N×r) was obtained with different amounts of total labeled aptamer added.

<table>
<thead>
<tr>
<th>Total aptamer concentration [nM]</th>
<th>Bound aptamer number in the confocal volume (N×r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1.9±1.5</td>
</tr>
<tr>
<td>0.3</td>
<td>16.8±4.6</td>
</tr>
<tr>
<td>0.5</td>
<td>34.1±4.4</td>
</tr>
<tr>
<td>0.7</td>
<td>46.6±5.3</td>
</tr>
<tr>
<td>1.0</td>
<td>59.5±4.5</td>
</tr>
<tr>
<td>1.2</td>
<td>67.9±2.7</td>
</tr>
<tr>
<td>1.4</td>
<td>74.4±3.7</td>
</tr>
<tr>
<td>1.6</td>
<td>82.1±3.0</td>
</tr>
<tr>
<td>2.0</td>
<td>85.5±1.3</td>
</tr>
<tr>
<td>2.5</td>
<td>85.8±0.9</td>
</tr>
<tr>
<td>3.0</td>
<td>86.0±0.7</td>
</tr>
</tbody>
</table>
Table 2
Comparison of the $K_d$ value of IgE–receptor complexes and the IgE receptor density on RBL-2H3 cells as determined by using different methods.

<table>
<thead>
<tr>
<th>Method for $K_d$ determination</th>
<th>Dissociation constant $K_d$ [nM]</th>
<th>IgE receptor density [receptor cell$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>fluorescence quenching$^{[18]}$</td>
<td>0.71</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>FCS</td>
<td>$0.81 \pm 0.21$</td>
<td>$2.88 \times 10^5 \pm 1200$</td>
</tr>
</tbody>
</table>