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A Sensitive and Microscale Method for Drug Screening Combining Affinity Probe and Single Molecule Fluorescence Correlation Spectroscopy

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ABSTRACT: In this paper, a sensitive and microscale method for drug screening is described using single molecule spectroscopy fluorescence correlation spectroscopy (FCS). The principle of this method is mainly based on the competition of candidate drugs to the fluorescent probe-target complexes and the excellent capacity of FCS for sensitively distinguishing the free fluorescent probes and the fluorescent probe-target complexes in solution. In this study, the screening of protein kinase inhibitors was used as a model, tyrosine-protein kinase ABL1 was used as a target and a known inhibitor dasatinib derivative labeled with fluorescent dye was used as a fluorescent affinity probe. We firstly established the theoretical model of drug screening based on the binding process of fluorescent probes and targets, the competition of candidate drugs to the fluorescent probe-target complexes and FCS theory. And then, the dasatinib derivatives were synthesized and labeled with fluorescent dye Alexa 488, the binding and dissociation processes of Alexa 488-dasatinib and ABL1 were systematically investigated. The dissociation constant and dissociation rate for Alexa 488-dasatinib-ABL1 complex were determined. Finally, the established method was used to screen candidate drugs. The dissociation constants of ABL1 kinase to six known drugs for treating chronic myeloid leukemia (CML) were evaluated and the results obtained are well consistent with the reported values. Furthermore, a homemade chip with micro-wells was successfully utilized in FCS measurements as the carrier of samples, and the sample requirements were only 1~2 μL in this case. Our results demonstrated that the drug screening method described here is universal, sensitive and small sample and reagent requirement. We believe that this method will become a high throughput platform for screening of small molecule drugs.

Introduction

The development of new drugs is very important to improvement of human health due to the rapid appearance of numerous single, multiple, and extensively drug-resistant forms of the diseases.[1] The new drug discovery relies on massive screening of natural and synthetic compound libraries against various extracellular and intracellular molecular targets,[2,3] which requires a major investment of capital and human resources. In the new drug discovery and development, one key issue is how to develop new methods to dramatically reduce the cost and time of drug screening.[4] Currently, some technologies and methods were developed for drug screening, and they mainly include microfluidics,[5,6] microarray,[7] mass spectrometry (MS),[8] nuclear magnetic resonance (NMR) spectroscopy,[9-11] fluorescence methods,[12-14] surface plasmon resonance (SPR),[15]capillary electrophoresis,[16,17] and flow cytometry.[18] Rosenthal *et al.* reported a fluorescence displacement method for antidepressant drug discovery based on ligand-conjugated quantum dot (QD).[19] Kang *et al.* developed a method for screening of kinase inhibitors by capillary electrophoresis.[16] Shi *et al.* designed and synthesized a new fluorescent light-up probe for real-time cell apoptosis imaging and apoptosis-inducing agents screening.[20] Yi *et al.* established a SPR-based and signal-amplified enzymatic assay for facile inhibitor screening.[21] Cravatt's group proposed a new screening method for identification of selective inhibitors of uncharacterized enzymes using fluorescent activity-based probes.[22] Although the development of those new assays have made great progresses in drug screening, our main challenge is how to markedly reduce the sample and reagent requirements to lower the cost of drug screening especial for some expensive target proteins and enzymes. Fluorescence correlation spectroscopy (FCS)[14,23-25] is a single molecule method with the fL level detection volume. FCS has been rapidly developing in recent years. A few variants of FCS have been developed,[26] such as fluorescence cross-correlation spectroscopy (FCCS),[27] scanning FCS (SFCS),[28] image fluorescence correlation spectroscopy (IFCS),[29] single plane illumination microscopy FCS (SPIM-FCS),[30] stimulated emission depletion FCS (STED-FCS)[31] and resolved scattering correlation spectroscopy (RSCS).[32] FCS becomes a sensitive and efficient tool for homogenous assays,[33-35] studying molecular interaction both *in vitro*[36] and *in vivo*.[37] Kawaguchi *et al.* explored the possibility for screening of ASK1 kinase inhibitors by FCS technique.[38] Mikuni *et al.* developed a FCS based method for studying the protein–peptide interaction and inhibition.[39] Recently, our group developed a sensitive method for the assay of drug-induced apoptosis by FCS technique.[40] Although FCS shows a great potential for drug screening, to date, its applications are very difficultly expanded due to high expensive commercial FCS instruments and shortage of the corresponding methods and theoretical models of drug screening.

In this paper, we described a sensitive and microscale method for drug screening combining FCS technique with the competition reaction of candidate drugs to the fluorescent probe-target complexes. Our motivations are: (1) to establish the competitive theoretical model of FCS-based drug screening; (2) to dramatically reduce the sample and reagent requirements combining FCS technique and PDMS/glass chip with microwells. In this study, the screening of tyrosine-protein ABL protein kinase inhibitors was used as a model, ABL1 was used as a target, and a known inhibitor dasatinib[41] labeled with fluorescent dye was used as a probe. BCR-ABL is a constitutively activated tyrosine kinase that causes chronic myeloid leukemia (CML),[42] a specific inhibitor of the ABL

protein tyrosine kinase might be an effective and selective therapy for CML and other BCR-ABL-positive leukemias.[43] Firstly, we established the theoretical model of drug screening, and then, designed and synthesized dasatinib derivatives labeled with fluorescent dye Alexa 488 as a fluorescent affinity probe. Finally, the FCS based drug screening method was established and was successfully used to evaluated the dissociation constants of six known CML drugs (dasatinib, imatinib,[44] nilotinib,[45] sunitinib,[46] gefitinib[47] and erlotinib[48]) to ABL1 kinase.

Theoretical section

The principle of the drug screening method based on FCS technique is shown in Figure 1, and it is mainly based on the competition of candidate drugs to the fluorescent probe-target complexes and the capacity of FCS for sensitively distinguishing the free fluorescent probes and the fluorescent probe-target complexes in solution. Firstly, an active compound (known drug or affinity protein profiling probes), which can bind to the target molecules, is labeled with fluorescent dye as probes. In the presence of the target molecules, fluorescent probes can bind to the target molecules to form the fluorescent probes-target complexes. And then, candidate compounds are added to the fluorescent probe-target complexes solution. If the candidate compounds and the probes share the same binding site on the target molecules, certain fluorescent probes will be replaced due to competition reaction of the candidate compounds. Finally, FCS is applied to evaluate the dissociation constants of candidate compounds using the competitive reaction fitting model.

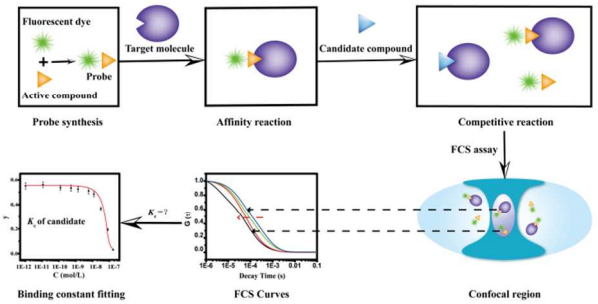

Figure 1. The principle of drug screening method based on FCS technique.

FCS Theory and Data Processing

FCS is a single molecule method, and its principle is based on measuring the fluorescence fluctuations in a small highly focused detection volume (less than 1fL) due to Brownian motion of single fluorescent molecules. In this method, FCS is applied to distinguish the free fluorescent probes and the fluorescent probe-target complexes in solution due to the significant difference in their diffusion coefficients. In FCS measurement, the fluorescent intensity $F(t)$ is recorded, the time-dependent fluorescence fluctuations $\delta F(t)$ around the mean values are thus obtained and these fluctuations are autocorrelated in time. The autocorrelation function $G(\tau)$ is defined as

$$G(\tau)=\frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} \quad (1)$$

where the symbol $\langle\rangle$ stands for the ensemble mean value and τ is the time delay. If a three-dimensional Gaussian profile in the focal volume is assumed and the triplet state of a fluorophore was considered, the equation (1) can be expressed as

$$G_D(\tau)=\left(1+\frac{Te^{-\tau/\tau_{tr}}}{1-T}\right) \times\sum_{i=1}^{n}\frac{\tau_{Di}}{N_i(\tau_{Di}+\tau)}\times\frac{1}{\sqrt{1+(\frac{\omega_0}{Z_0})^2\times\frac{\tau}{\tau_{Di}}}} \quad (2)$$

Where T is the fraction of the fluorescent particles in triplet state whose lifetime is τ_{tr}, N_i and τ_{Di} denote the number of fluorescent molecules in the detection volume and the characteristic diffusion time of component i, respectively. The characteristic diffusion time (τ_i) of the i_{th} component is defined as

$$\tau_i=\frac{\omega_0}{4D_i} \quad (3)$$

where diffusion coefficient (D_i) can be obtained by calibrating with the standard substance (such as Rhodamine Green (RG)).

$$D_i=\frac{\tau_{RG}}{\tau_i}\times D_{RG} \quad (4)$$

When n=1, the equation (2) can be changed into the single component model as following

$$G(\tau)=\frac{1}{\langle N\rangle}\times(1+\frac{Te^{-\tau/\tau_{tr}}}{1-T}) \times\frac{1}{(1+\frac{\tau}{\tau_D})}\times\frac{1}{\sqrt{1+(\frac{\omega_0}{z_0})^2\times\frac{\tau}{\tau_D}}} \quad (5)$$

All raw FCS data were analyzed with the standard equation for multi-component model in equation (2) and nonlinearly fitted with the Microcal Origin 8.0 software package based on the Levenberg-Marquardt algorithm. The detection volume of FCS system was measured using a RG solution with the concentration of 2.0 nM (its diffusion coefficient: 2.8×10^{-6} cm^2/s in water) as a standard substance. The detection volume obtained was about 0.4 fL.

Binding Reaction of Fluorescent Probes and Targets

The binding reaction of Alexa 488-Dasatinib (**A**) to ABL1 protein (**P**) can be described as

$$A+P\rightleftharpoons AP \quad (6)$$

The dissociation constant (K_d) can be expressed as

$$K_d = \frac{[A][P]}{[AP]} \quad (7)$$

The percentage of the **AP** complex (y) can be acquired with FCS, and is defined as

$$y = \frac{[AP]}{[A]+[AP]} \quad (8)$$

where [**A**] and [**P**] are the concentrations of free **A** and **P** respectively, [**AP**] is the concentration of **AP** complex. Let $[A]_0$ and $[P]_0$ stand for the initial concentrations of **A** and **P**, and we obtained:

$$[A]_0 = [A]+[AP] \quad (9)$$

$$[P]_0 = [P]+[AP] \quad (10)$$

Substituting the equation (7) into the equations (8), y can be modified as

$$y = \frac{K_d + [A]_0 + [P]_0}{2[A]_0} + \sqrt{\left(\frac{K_d + [A]_0 + [P]_0}{2[A]_0}\right)^2 - \frac{[P]_0}{[A]_0}} \quad (11)$$

The y can be obtained by two-component model of FCS. In the two mutually reactive components model, when two components diffuse through the detection volume with the characteristic diffusion time τ_{free} and τ_{bound} , the equation (2) can be modified as the following equation

$$G(\tau) = \frac{1}{N} \times \left(1 + \frac{Te^{-\tau/\tau_{tr}}}{1-T}\right) \times \left[\frac{1-y}{\left(1+\frac{\tau}{\tau_{free}}\right)\sqrt{1+(\frac{\omega_0}{Z_0})^2 \times \frac{\tau}{\tau_{free}}}} + \frac{y}{\left(1+\frac{\tau}{\tau_{bound}}\right)\sqrt{1+(\frac{\omega_0}{Z_0})^2 \times \frac{\tau}{\tau_{bound}}}}\right] \quad (12)$$

The τ_{free} and τ_{bound} are the characteristic diffusion times of the free fluorescent probe and the binding complex. Binding data were analyzed with the simple binding model based on a stoichiometry of one fluorescent probe per each acceptor. Fitting was carried out with Microcal Origin 8.0 software. We initially plotted the y , which was obtained by FCS measurement as described above, against the concentration of ABL1 (x). For all evaluations of K_d, this titration curve was fitted with equation (11) based on Levenberg-Marquardt algorithm.

Competitive Reaction of Candidate Compounds

Dissociation constant values of competitive compounds (K_c) were determined from a competition experiment, in which serial dilutions of protein kinase inhibitor drugs (**C**) were added to a fixed concentration of the **AP** complex. Due to the concentration depletion of all three starting species (**A**, **P** and **C**) in our competition experiment, the equilibrium concentrations of all five species (**A**, **P**, **C**, **AP** and **CP** complex) were calculated by solving a cubic equation and using the physiologically meaningful solution.[49] According to the references about competitive assays,[49,50] we can derive the K_c value expression in a competitive mode based on FCS technique. The derivation and solution of a cubic equation described elsewhere follow as

$$A + P \rightleftharpoons AP \quad (6)$$

$$C + P \rightleftharpoons CP \quad (13)$$

$$K_d = \frac{[A][P]}{[AP]} \quad (7)$$

$$K_c = \frac{[C][P]}{[CP]} \quad (14)$$

$$[C]_0 = [C]+[CP] \quad (15)$$

$$[P]_0 = [P]+[AP]+[CP] \quad (16)$$

$$[AP] = \frac{[P][A]_0}{K_d + [P]} \quad (17)$$

$$[CP] = \frac{[P][C]_0}{K_c + [P]} \quad (18)$$

Substituting equation (17) into equation (8), we have

$$y = \frac{[P]}{K_d + [P]} \quad (19)$$

Solving the proper root of [**P**] according to the reference[49] (the detailed derivation process was presented in the Supplementary Information), we can obtain

$$[P] = -\frac{a}{3} + \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\frac{\theta}{3} \quad (20)$$

Where a, b, c and θ are related to $[A]_0$, $[P]_0$ and $[C]_0$, and they are expressed as the following

$$a = K_d + K_c + [A]_0 + [C]_0 - [P]_0$$

$$b = K_d([C]_0 - [P]_0) + K_c([A]_0 - [P]_0) + K_d K_c$$

$$c = -K_d K_c [P]_0$$

$$\theta = \arccos\frac{-2a^3 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}}$$

K_d can be calculated from the **A** and **P** binding reaction as described in equation (11). The equation (19) will be changed into the relationship with [**P**] and K_c. Since $[A]_0$, $[P]_0$, $[C]_0$ and K_d are known in this case, the equation (19) containing K_c can be simplified as

$$y = f(K_c) \quad (21)$$

The y is determined from each titration point in FCS measurement with the fixed **C** concentration using the equation (12).The titration curve is fitted with the equation (21) using Levenberg-Marquardt algorithm of the Microcal Origin 8.0 software, and dissociation constant K_c for different inhibitors to ABL1 protein can be obtained.

Experimental section

Materials

Rhodamine Green and Alexa Fluor® 488 Succinimidyl Ester (Alexa 488) were purchased from Life Technologies Inc. Dasatinib, nilotinib, imatinib, erlotinib, sunitinib and gefitinib were purchased from Nanjing Ange Pharmaceutical Co., Ltd. (China). Tyrosine-protein kinase ABL1 protein (ABL1) was obtained from Sino Biological Inc. (China). Boc-6-aminohexanoic acid (Boc-6-Ahx-OH), diethyl azodicarboxylate (DEAD) and triphenylphosphine (PPh3) were purchased from Aldrich-Chemie. All other chemicals used in this study were of analytical grade and used without further purification. All solutions were prepared with ultrapure water purified on Millipore simplicity.

Synthesis of Alexa 488-dasatinib

The procedure for the synthesis of Alexa 488-dasatinib (**A**) is demonstrated in Figure 2. Experimental details are described in Supplementary Information.

Apparatus

FCS measurements were performed with a home-built FCS system,[51] and the setup of FCS is shown in Figure S1 in Supplementary Information. This setup was based on an inverted Olympus IX 71 microscope (Japan). The illumination source was an argon laser with 488 nm laser line (Ion Laser Technology, Shanghai, China). The dichroic mirror was 505DRLP (Omega Optical) and the band pass filters was 530DF30 (Omega Optical).And the fluorescence fluctuations were correlated with a digital correlator (Flex02–12D/C, Correlator.com). FCS measurements were carried out over a period of 60 s at room temperature (about 25 ^{o}C), and were repeated 3 times. The procedure for fabrication of the PDMS/glass chip with micro-wells was provided in Supplementary Information.

Binding Assay by FCS

To a solution of Alexa 488-dasatinib (final concentration 2 nM) in PBS (pH 7.4), an equal volume of various concentrations of ABL1 1, 10, 20, 40, 50, 100, 200, 400, 500 nM were added. The mixture was incubated at 25 ^{o}C for 1 hr. And then, the samples were measured by FCS.

Competition Assay by FCS

To a solution of Alexa 488-dasatinib (final concentration 2 nM) in PBS (pH 7.4), and equal volume of ABL1 in PBS (final concentration 50 nM) were added. The mixture was incubated at 25 ^{o}C for 1 hr. Appropriate various concentrations of dasatinib, nilotinib, imatinib, erlotinib, sunitinib and gefitinib were added. The mixture was incubated at 25 ^{o}C for 1 hr. And then, the samples were measured by FCS.

Results and discussion

Fluorescent Probe and Its Stoichiometric Ratio to ABL1 Protein

The optical and chemical properties of fluorescent affinity probes (fluorescent probes) are very important in this new method. The ideal fluorescent probe should possess certain binding capacity to target molecules, good photo stability and high brightness per molecule (BPP) in FCS measurements. In this study, dasatinib derivatives labeled with Alexa 488 dye are used as a fluorescent probe because dasatinib can strongly bind to ABL1 protein and Alexa 488 dyes show excellent photo stability, good water-solubility and high fluorescent quantum yield. The synthesis of dasatinib derivatives and labeling of Alexa 488 dye are demonstrated in Figure 2. In the design of the fluorescent probe, an "arm" (compound **2**) is introduced to increase the distant between Alexa 488 and dasatinib in order to retain the good binding capacity of dasatinib and prevent the fluorescence quenching of Alexa 488 dyes. The experimental details are described in Supplementary Information. Characterizations of compound **4** and Alexa 488-dasatinib compound **A** were performed by mass spectrometry. These data shown in Figure S2 illustrate the successful synthesis of Alexa 488-dasatinib compounds.

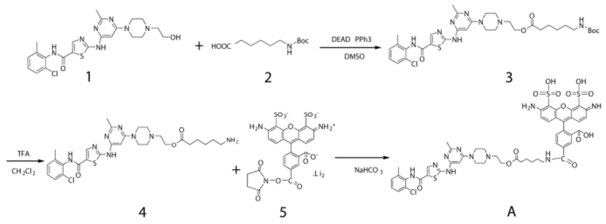

Figure 2. The procedure for synthesis of Alexa 488-dasatinib (**A**).

Typical FCS curves of **RG**, Alexa 488, Alexa 488-dasatinib (**A**) and Alexa 488-dasatinib-ABL1 complex (**AP**), their fitting curves and corresponding fit residuals are shown in Figure 3a. FCS curves of four compounds are well fitted with the single-component model equation (5) with correlation coefficients (R^2) of 0.990~0.997, and the fitting residuals are less than 0.05. The obtained diffusion coefficients of Alexa 488, free Alexa 488-dasatinib and its ABL1 complex were determined as (2.61±0.09) × 10^{-6} cm^2/s, (2.53±0.07) × 10^{-6} cm^2/s and (0.72±0.04) × 10^{-6} cm^2/s, respectively. These results show the significant difference between the diffusion coefficient of free **A** and **AP** complex, and therefore, FCS can be used to distinguish the free Alexa 488-dasatinib and its ABL1 complex in the mixed solution. Furthermore, we observed that the BPP values of Alexa 488, Alexa 488-dasatinib and its ABL1 complex were very similar in Figure 3b. These results illustrated: (1) the labeling of dasatinib with Alexa 488 was very successful because dasatinib molecules did not quench the fluorescence of Alexa 488; (2) the stoichiometry of this binding reaction was 1:1 due to nearly same BPP values of Alexa 488-dasatinib and its ABL1 complex.

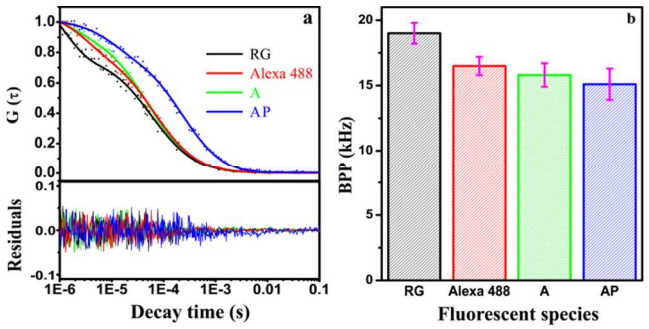

Figure 3. (a) The auto correlation curves, fitting curves and their fitting residuals of Rhodamine Green, Alexa 488，Alexa 488-dasatinib and Alexa 488-dasatinib-ABL1 protein complex. (b) Their brightness per molecule (BPP) was obtained by FCS. The laser intensity is 80 μW, and the concentration of each dye is 1 nM. The measurement time was 60 s. The error bars represent the standard deviation of 3 time measurements. **RG**: Rhodamine Green, **A**: Alexa 488-dasatinib, **AP**: Alexa 488-dasatinib-ABL1 complex.

Dissociation Rate and Dissociation Constant of Alexa 488-dasatinib

Firstly, we observed the binding process between Alexa 488-dasatinib and ABL1. The results shown in Figure 4a illustrated that the binding of fluorescent probe to protein was a fast kinetic process, and within 45 min the binding reaction reached the equilibrium in this case. Therefore, we chose the incubation time as 1 hr at room temperature. We previously reported the real time and on-line observation of the immune reaction process by FCS technique.[34] Similarly, FCS is used to investigate the dissociation of Alexa 488-dasatinib-ABL1 complex in the presence of dasatinib. The dissociation rate was determined by using competitive displacement mode. Initially, the Alexa 488-dasatinib (1 nM) was mixed with ABL1 (500 nM) in PBS, incubated for 60 min at room temperature, and then 50 nM of the unlabeled dasatinib was added to replace Alexa 488-Dasatinib. Autocorrelation curves were collected every 10 s for the first 300 s, and then the collection interval was increased to 1 min in the remaining time. The data were shown in Figure 4b and fit by using the model we previously reported: $y = \exp(-k_{off} \times t) + D$,[52] where k_{off} is the dissociation rates, and ***D*** denotes a constant offset. The result obtained was k_{off} = (1.8±0.1) × 10^{-3} S^{-1}, and constant ***D*** = (2.4±0.1) × 10^{-2}. The data illustrate that the reaction rate is very fast, and is well suitable for developing a rapid competitive assay by FCS technique.

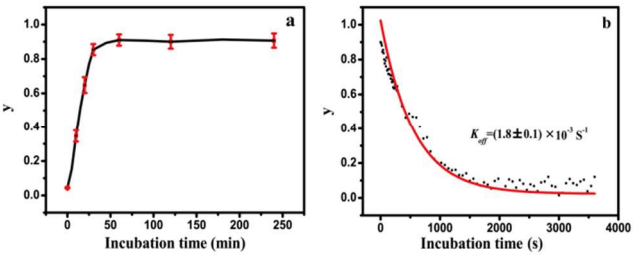

Figure 4. The binding and dissociation kinetic curve of Alexa 488-dasatinib to ABL1 complex. (a) The binding rate curve: 1 nM Alexa 488-dasatinib was mixed with 500 nM ABL1 in PBS, incubated at room temperature, after incubation of 15, 30, 45, 60, 120, 240 min, and measured by FCS, respectively. The measurement time was 60 s. The error bars represent the standard deviation of 3 time measurements. (b) The dissociation kinetic curve: the monitoring time of the reaction is 3600 s. The data were fitted using a single-exponential rate model. The off-rate is (1.8±0.1) × 10^{-3} S^{-1}.

Figure 5a shows the normalized fluorescence correlation curves of Alexa 488-dasatinib and its ABL1 complex, these autocorrelation curves are well fitted with the theoretical mode of autocorrelation function as described in the equation (12) with correlation coefficients (R^2) of 0.992 ~ 0.998. The fitting residuals as shown in Figure 5b are very low (less than 0.05). A fit with equation (11) gave a value for the dissociation constant at room temperature. The titration curve is shown in Figure 5c, the dissociation constant K_d is (4.0±0.25) × 10^{-8} mol/L, and its correlation coefficient (R^2) is 0.993. These data illustrated that the fluorescent probe used in this study had a good affinity to ABL1 protein, which further illustrated that the design and preparation of Alexa 488-dasatinib probes were successful.

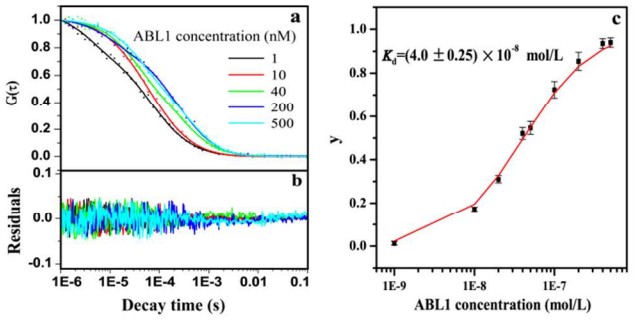

Figure 5. (a) Normalized fluorescence correlation curves of Alexa 488-dasatinib and its binding complex. ABL1 (concentration increased from 1 nM to 500 nM) were titrated into Alexa 488-dasatinib solution kept at a fixed concentration (1 nM). (b) The fitting residuals. (c) Titration curve obtained from FCS measurements. The faction of the binding complex was

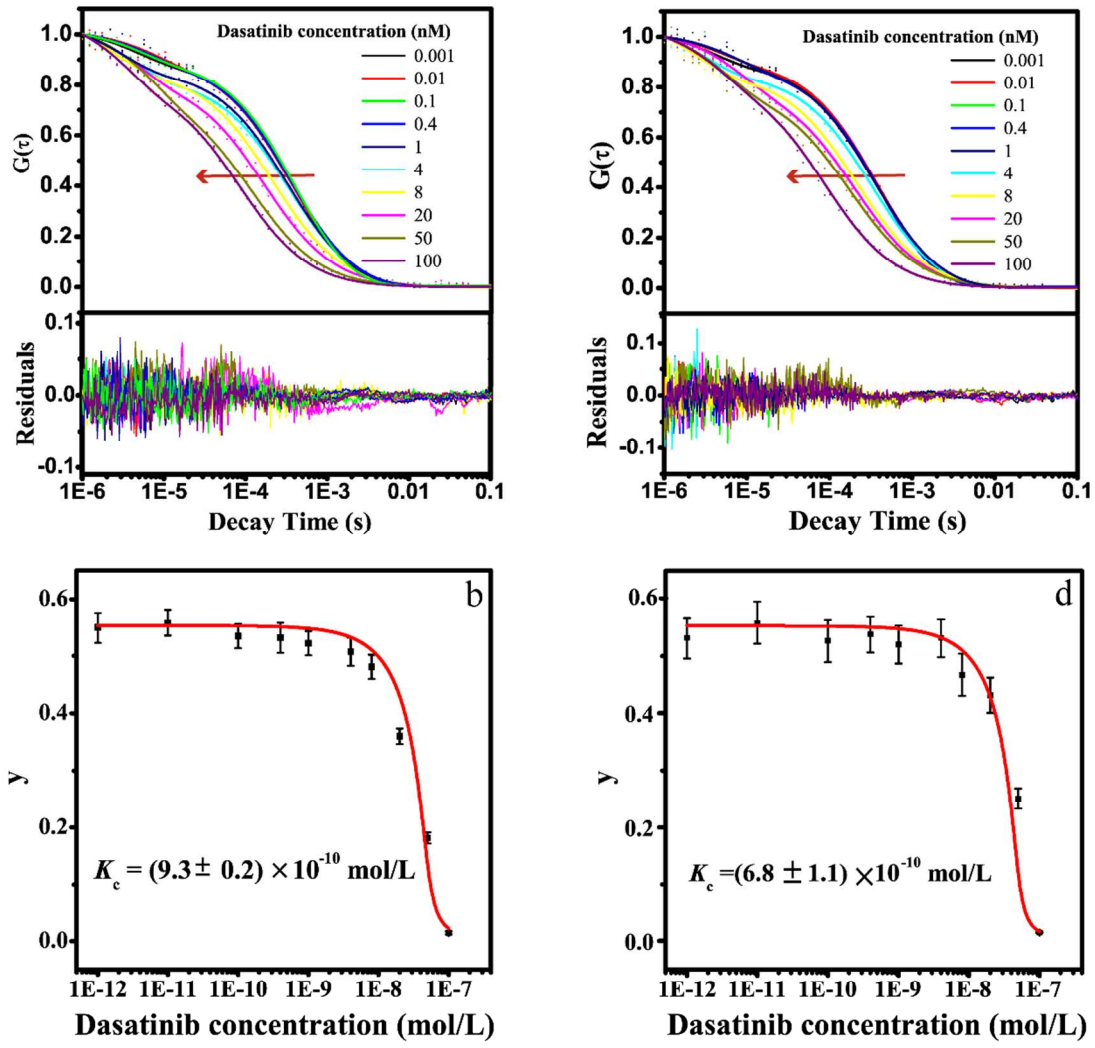

Figure 6. The autocorrelation, fitting curves and their fitting residuals of dasatinib competitive reaction solution measured on a coverslip (a) and a homemade chip with micro-wells (c). Titration curves of dasatinib competitive binding to ABL1 using a coverslip and a homemade chip with micro-wells are shown in (b) and (d), respectively. The faction of the affinity complex was potted as function ofdasatinibconcentration1.0 × 10^{-12} mol/L ~1.0 × 10^{-7} mol/L. The dissociation constant (K_c) of dasatinib to ABL1 assayed on coverslip and micro-well was （9.3±0.2） × 10^{-10} mol/L and （6.8±1.1） × 10^{-10} mol/L, respectively. The measurement time was 60 s. The error bars represent the standard deviation of3 time measurements.

Dissociation Constants of Inhibitors

Dissociation constants of different inhibitors can be obtained according to the competitive reactions described above. Theoretically, the percentage of Alexa 488-dasatinib-ABL1 complexes (y) will decline when the different concentrations of competitive compounds are added into Alexa 488-dasatinib-ABL1 complex solution. The autocorrelation and fitting curves of dasatinib competitive reaction and their corresponding fit residuals are shown in Figure 6a. In this case, 20 μL samples were put on a coverslip. FCS curves of different dasatinib concentrations are well fitted with the two mutually reactive components model with correlation coefficients (R^2) of 0.986~0.998, and the fitting residuals are less than 0.07. Figure 6b shows the titration curve of dasatinib competitive reaction obtained from FCS measurements. The titration curve was well fitted with equation (21), and its correlation coefficient (R^2) was 0.987. The obtained dissociation constant K_c was (9.3±0.2) × 10^{-10} mol/L, which is well consistent with the reported value (5.3 × 10^{-10} mol/L). [53]

In order to reduce the requirements of samples and reagents, a fabrication of PDMS/glass chip with micro-wells was used as a sample carrier in FCS measurements. The procedure for fabrication of PDMS/glass chip with micro-wells was described in Supplementary Information. This chip contains 36 micro-wells and each well can fill ~2 μL sample. The auto correlation curves of free Alexa 488, Alexa 488-dasatinib and Alexa 488-dasatinib-ABL1 protein complex measured on the chip with micro-wells were provided in Figure S6, The FCS curves are in reasonable agreement with those on the co-

verslip, which illustrated that Alexa 488, Alexa 488-dasatinib and Alexa 488-disatinib-ABL1 complex were not adsorbed on PDMS/glass chip with micro-wells. Figure 6c shows the auto-correlation and fitting curves of dasatinib competitive reaction assayed on micro-wells and their corresponding fit residuals. FCS curves of different concentrations dasatinib are well fitted with the two reactive components model with their correlation coefficients (R^2) of 0.979~0.996, and the fitting residuals are less than 0.12. Figure 6d shows the titration curve of dasatinib competitive binding to ABL1 obtained by micro-wells. The titration curve was well fitted with equation (21) as well, and R^2 was 0.967. The fitted dissociation constant K_c was (6.8±1.1) $\times 10^{-10}$ mol/L, which is well consistent with the value obtained with the coverslip.

We investigated the reliability of this method using dasatinib as a model sample, and the relative standard deviations (RSDs) of K_c are 6.7% (within-day, n=5) and 9.8% (between-day, n=3), respectively. Our preliminary results documented that this method had a good reproducibility. Furthermore, this method is applied to measure the dissociation constants of other five known CML drugs, and the structures of these drugs are shown in Figure S3. The titration curves of inhibitor competitive reactions were provided in Figure S4. These curves were well fitted with the equation (21) and their correlation coefficients (R^2) were from 0.936 to 0.991. Table 1 shows the fitted dissociation constants of six CML drugs, and the results obtained were in good agreement with the reported values. As shown in Table 1, this method was successfully used to measure the dissociation constants of inhibitors from 4.6 $\times$ 10^{-6} mol/L (low) to 9.3 $\times 10^{-10}$ mol/L (high). These results indicate that our method has a good universality, and is suitable for screening of protein kinase inhibitors and other protein target binding drugs.

Table 1. Dissociation constants of six CML drugs obtained by FCS method and their reported values

Compound	Dissociation constant (mol/L) FCS result	Reported value	Reference
dasatinib	(9.3±0.2) $\times 10^{-10}$	5.3×10^{-10}	
erlotinib	(4.0±0.1) $\times 10^{-7}$	3.1×10^{-7}	53[a]
sunitinib	(8.0±0.8) $\times 10^{-7}$	8.3×10^{-7}	
nilotinib	(5.6±1.6) $\times 10^{-9}$	$<3.0\times10^{-8}$	54[b]
imatinib	(9.0±0.8) $\times 10^{-9}$	1.4×10^{-8}	55[c]
gefitinib	(4.6±0.6) $\times 10^{-6}$	$>1.0\times10^{-6}$	56[a]

The letters indicate methods for determination of inhibitors' dissociation constants in references, *a*: quantitative PCR, *b*: immuno-precipitation and *c*: spectrophotometry.

Conclusions

In this paper, we proposed a sensitive and microscale method for drug screening using fluorescence correlation spectroscopy, and established the corresponding theoretical model of drug screening based on the binding process of fluorescent probes and targets, the competition of candidate drugs to the fluorescent probes-target complexes and FCS theory. In this study, the screening of protein kinase inhibitors was used as a model, tyrosine-protein kinase ABL1 protein was used as a target and a known inhibitor dasatinib labeled with fluorescent dye was used as a probe. We designed and synthesized the Alexa 488-dasatinib compound as a fluorescent probe, and studied the binding reaction of Alexa 488-dasatinib and ABL1 protein and the competitive reaction of candidate drugs to Alexa 488-dasatinib-ABL1 protein complexes. The dissociation constants of candidate drugs can be obtained by the theoretical model. The established method was successfully used to evaluate the dissociation constants of six known CML drugs, and the results obtained were well consistent with the reported values. Compared to current methods, our method is characterized as simplicity, high sensitivity, good universality and small sample and reagent requirements. More importantly, the detection volume of FCS is less than 1fL and the sample requirement can easily be reduced to nL level using a droplets array technique, which remarkably reduce the cost of drug screening in the future. Therefore, our method has the potentiality to become a high throughput platform for screening of protein kinase inhibitors and other protein target binding drugs using active protein profiling probes.

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Notes and references

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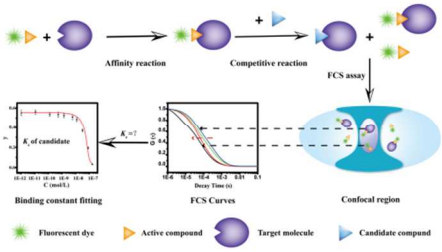
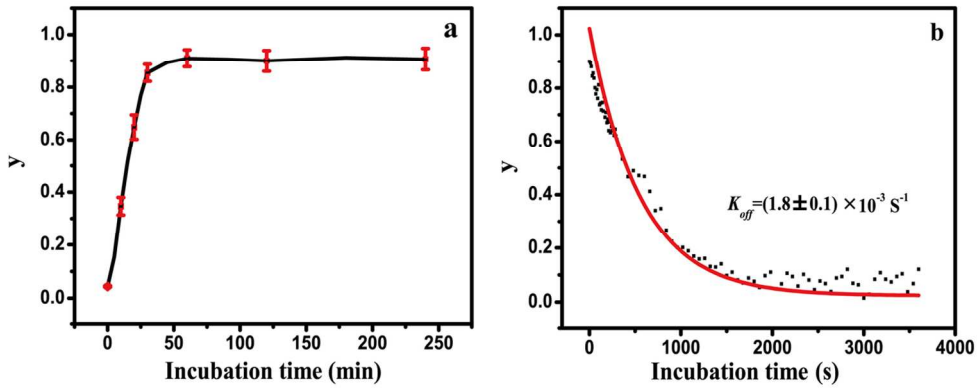


The technical route of drug screening method based on competitive reaction and fluorescence correlation spectroscopy.



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