Chapter 30

SH2 Domain-Based FRET Biosensor for Measuring BCR-ABL Activity in Living CML Cells

Mari Fujioka, Yumi Asano, Shigeyuki Nakada, and Yusuke Ohba

Abstract

Fluorescent proteins (FPs) displaying distinct spectra have shed their light on a wide range of biological functions. Moreover, sophisticated biosensors engineered to contain single or multiple FPs, including Förster resonance energy transfer (FRET)-based biosensors, spatiotemporally reveal the molecular mechanisms underlying a variety of pathophysiological processes. However, their usefulness for applied life sciences has yet to be fully explored. Recently, our research group has begun to expand the potential of FPs from basic biological research to the clinic. Here, we describe a method to evaluate the responsiveness of leukemia cells from patients to tyrosine kinase inhibitors using a biosensor based on FP technology and the principle of FRET. Upon phosphorylation of the tyrosine residue of the biosensor, binding of the SH2 domain to phosphotyrosine induces conformational change of the biosensor and brings the donor and acceptor FPs into close proximity. Therefore, kinase activity and response to kinase inhibitors can be monitored by an increase and a decrease in FRET efficiency, respectively. As in basic research, this biosensor resolves hitherto arduous tasks and may provide innovative technological advances in clinical laboratory examinations. State-of-the-art detection devices that enable such innovation are also introduced.

Key words Förster resonance energy transfer (FRET), Fluorescent protein (FP), Chronic myeloid leukemia (CML), BCR-ABL, Molecular target drug, CrkL, Fluorescence microscopy, Fluorescence cytometry (FCM), Fluorescence lifetime, Src Homology 2 (SH2) domain

1 Introduction

Green FP (GFP) was originally isolated from the luminous organ of the jellyfish *Aequorea victoria* by Dr. Osamu Shimomura [1]. It is indisputable that GFP has continued to shed light on cell biology since its cDNA was isolated in 1992 [2]. The primary reason why GFP is so revolutionary is its ability to be easily incorporated into cells via transfection, which is dependent on a key property of FPs, i.e., its ability to generate an intrinsic fluorophore without cofactors or enzymatic components [3]. It was not long before color variants of GFP were developed and FPs were distributed worldwide. Not only do these tools serve as markers of protein localization, but they are also used for monitoring intracellular

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environments under physiological conditions. In addition, based on the principles of the reconstitution of protein fragments or physicochemical energy transfer, protein–protein interactions or conformational changes can be monitored, widening the potential applications of FPs.

Recently, our research group has begun to expand the utility of FPs from basic biological research to the clinic [4]. As in basic research, FP-based biosensors have resolved hitherto arduous tasks and may provide innovative technological advances in clinical laboratory examinations. Specifically, we can now detect a minor population of drug-resistant cells and predict the most effective drug for each individual patient suffering from chronic myeloid leukemia (CML), which had been a utopian ideal for patients and clinicians for many years, using available clinical tests based on conventional concepts.

CML, a hematological malignancy involving the transformation of hematopoietic stem cells in bone marrow, is characterized by the formation of an abnormal chromosome (Philadelphia chromosome, Ph^1) and the expression of its transcript BCR-ABL [5]. BCR-ABL encodes a constitutively active tyrosine kinase that causatively contributes to the malignant transformation of leukemia cells by activating a range of signaling pathways via the tyrosine phosphorylation of its substrates, including SH2 containing proteins CrkL and signal transducer and activator of transcription (STAT) [6, 7]. The emergence of targeted therapy against BCR-ABL has radically changed the treatment of CML, which is now controllable using oral drugs. However, resistance and intolerance remain a concern for a substantial number of patients. Our FRET-based biosensor, referred to as phosphorylation indicator of CrkL *en* substrate (Pickles), may provide a promising future for such patients via the monitoring of drug responsiveness by accurately measuring BCR-ABL activity in living CML cells of from each patient (Fig. 1).

Pickles consists of a variant of yellow FP (YFP), the SH2 containing protein CrkL, and a variant of cyan FP (CFP) from the N-terminus. BCR-ABL phosphorylates CrkL on the tyrosine residue at position 207 (Y207), which induces binding of the SH2 domain and phosphorylated Y207 and thereby brings YFP close to CFP. Because FRET efficiency is inversely proportional to the sixth power of distance between a donor (CFP) and an acceptor (YFP), kinase activity and response to kinase inhibitors can be therefore monitored by an increase and a decrease in FRET efficiency, respectively. FRET efficiency can be determined by calculating the yellow-to-cyan emission ratio, which is conventionally provided by images acquired by fluorescence microscopy (Fig. 2).

Although fluorescence microscopy has been and continues to be the primary tool used to perform FRET experiments, the number of cells that can be observed within a certain period is restricted.

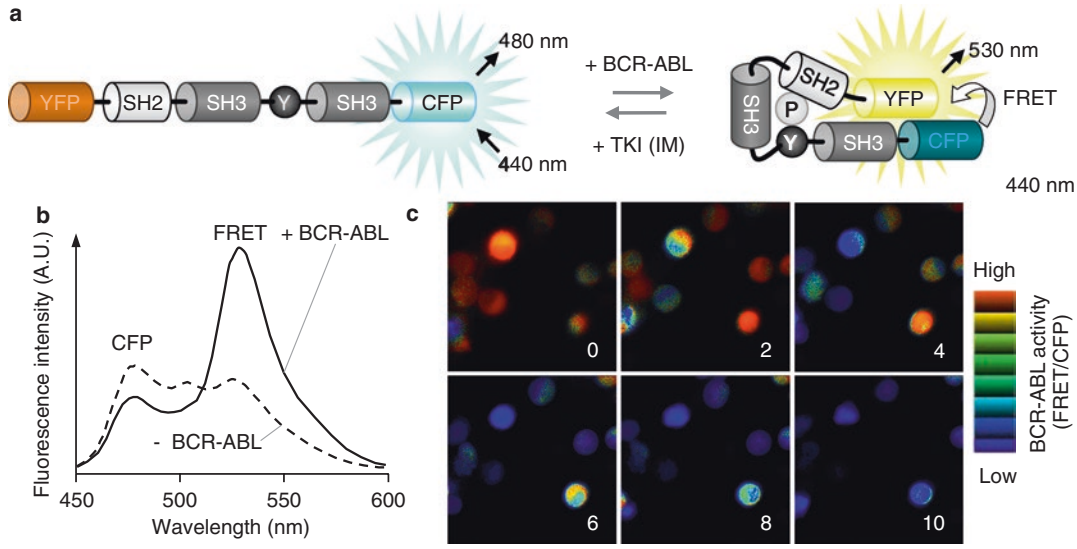

Fig. 1 The FRET-based biosensor Pickles to evaluate BCR-ABL activity in living CML cells. (**a**) Schematic representation of Pickle. The sandwiched region consists of one SH2 and two SH3 domains from human CrkL. P and Y represent a phosphate group and a tyrosine residue corresponding to Y207 of CrkL, respectively. Briefly, Pickles consists of a variant of YFP, CrkL, and a variant of CFP from the N-terminus. Upon Y207 phosphorylation, the SH2 domain binds to this phosphorylated tyrosine, which brings YFP close to CFP and increases the efficiency of FRET from CFP to YFP. (**b**) The fluorescence spectra of Pickles in the presence and absence of BCR-ABL. (**c**) K562 cells were transfected with the expression plasmid for Pickles, transferred to a glass base dish 24 h after transfection, and placed on an incubated stage equipped with a microscope. During time-lapse image acquisition, the cells were treated with IM. The photographs are representative FRET images in intensity-modulated display mode, a sort of pseudocolor mode in which the *red* and *blue colors* represent high and low FRET efficiency (BCR-ABL activity), respectively, at the indicated time point (h) after treatment

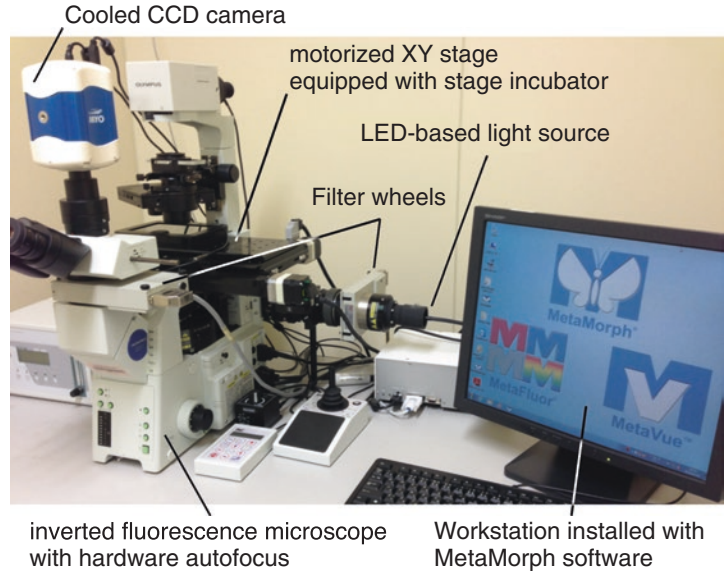

Fig. 2 Overview of the microscope and the peripherals used for data acquisition and analysis in this protocol. *CCD* charge-coupled device, *LED* light-emitting diode

Thus, a high throughput, quantitative FRET measurement technique is necessary to maximize the utility of the biosensor. Fluorescence flow cytometry is a good candidate for handling a vast number of cells in a short period; however, few studies are available regarding the successful application of this technique for the detection of FRET biosensors. The world's first flow cytometer designed to detect both the fluorescence lifetime and the fluorescence intensity [fluorescence lifetime cytometer (FLiCM, Fig. 3a)] might represent a suitable machine to improve the effectiveness and efficiency of present technologies.

The principle underlying the measurement of lifetime using the FLiCM are shown in Fig. 3b–d. The excitation light (laser), the output power of which is sinusoidally modulated at an angular frequency of ω ($2\pi \times 28$ MHz, amplitude modulation), is used to irradiate the sample that passes through the flow cell. The time-dependent component of the modulated laser power $P_E(t)$ is given by Eq. (1), where P_{E0} is the average power of the laser:

$$P_E(t) = P_{E0} sin\omega t. \tag{1}$$

The fluorescence intensity, $P_F(t)$, emanating from a fluorescent molecule that is irradiated by the laser is given by

$$dP_F(t) / dt = -(k_f + k_{nr}) P_F(t) + k_f N_0 P_E(t), \tag{2}$$

where k_f and k_{nr} represent rate constants for fluorescence and nonradiative transition, and N_0 is a factor dependent on the molar extinction coefficient and the concentration of the fluorescent molecule. From Eqs. (1) and (2),

$$P_F(t) = k_f N_0 \tau P_{E0} / \sqrt{1 + (\tau\omega)^2} \cdot \sin(\omega t - \theta) \tag{3}$$

$$\tau = (k_f + k_{nr})^{-1} \tag{4}$$

$$\theta = tan^{-1} \tau\omega, \tag{5}$$

where τ and θ are the fluorescence lifetime and phase shift, respectively. Furthermore, $k_f N_0 \tau P_{E0} / \sqrt{1 + (\tau\omega)^2}$ is the amplitude of the fluorescence intensity that sinusoidally varies over time, and $k_f N_0 \tau P_{E0}$ corresponds to the fluorescence intensity obtained by irradiation with the continuous-wave laser. Therefore, amplitude-modulated laser excitation results in fluorescence emission with a phase shift θ in a manner that is dependent on the substance-specific fluorescence lifetime τ, and fluorescence intensity can be determined based on its amplitude. To obtain the phase shift (θ) and the amplitude ($k_f N_0 \tau P_{E0} / \sqrt{1 + (\tau\omega)^2}$), $P_F(t)$ by $P_E(t)$ is

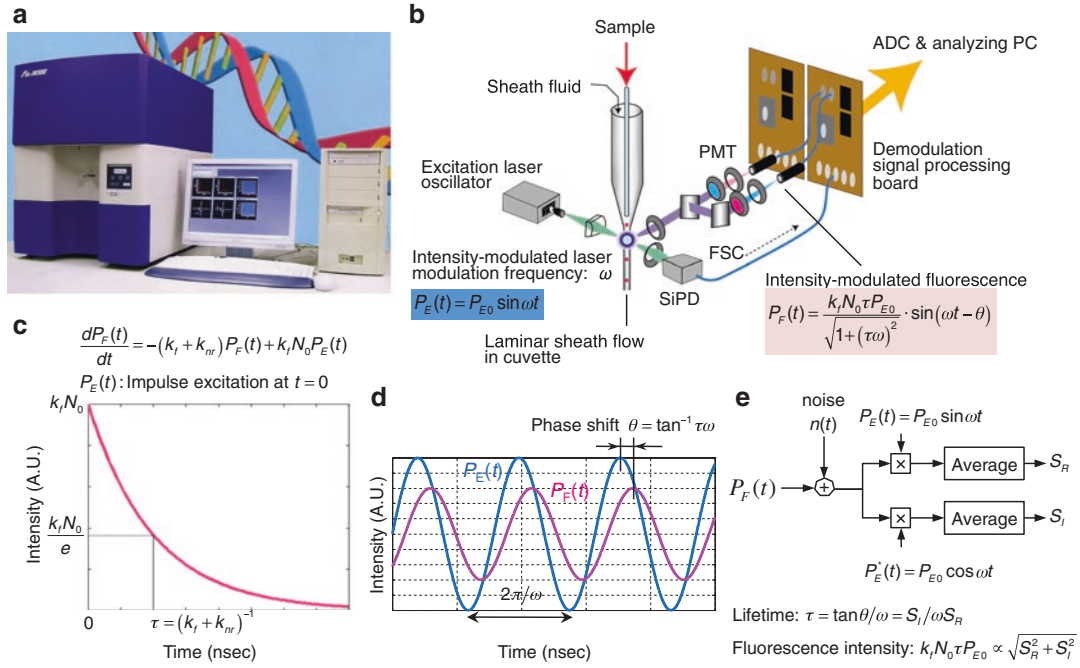

Fig. 3 The FLiCM and the principle of fluorescence lifetime measurement. (**a**) Exterior view of the FLiCM. The FLiCM system consists of the primary flow cytometer unit (*left side*) and a control/analyzing PC (*right side*). The primary FLiCM equipment includes an intensity-modulated laser, an optical system, a flow system, and a signal processing system for modulation and demodulation. FDS software, which is installed on the control/analyzing PC, manages data acquisition, graphical display, and the user interface. (**b**) The interior flow, optical paths, and the electronic circuits of the FLiCM. Samples of cells expressing FPs are carried to a cuvette via laminar sheath flow and intersect the laser beam orthogonally. FSC and SSC or fluorescence emission are detected using a SiPD and a PMT, respectively, and are transformed to electrical signals. The output electrical signals of fluorescence are demodulated in the signal processing circuits and are converted to the fluorescence lifetime or intensity in the control/analyzing PC. (**c**–**e**) Schematic representation of the principle of the measurement of fluorescence lifetime. The decay of the fluorescence intensity of a fluorescent molecule, the lifetime of which consists of a single component, that is excited by an impulse laser at $t=0$. The lifetime τ [s] of the fluorescence of the molecule is defined as the time at which the intensity decays by 1/*e*-fold (**c**). Modulated fluorescent emissions (*pink*) in response to modulated laser excitations (*blue*) at an angular frequency of ω. The phase shift θ [rad] in fluorescent emissions is a function of the fluorescence lifetime τ [s] (**d**). Schematic of the signal processing of the FLiCM. The modulated laser intensity $P_E(t)$ and the time-dependent fluorescence intensity $P_F(t)$ are demodulated to the real and imaginary signals S_R and S_I, respectively, from which the lifetime and the intensity of the fluorescence are determined (**e**)

demodulated, i.e., Eqs. (1) and (3) are multiplied, thereby obtaining real and imaginary portions of the demodulation signals, S_R *and* S_I, respectively:

$$S_R = k_f N_0 \tau P_{E0}^2 / 2\sqrt{1+(\tau\omega)^2} \cdot cos\theta \qquad (6)$$

$$S_I = k_f N_0 \tau P_{E0}^2 / 2\sqrt{1+(\tau\omega)^2} \cdot sin\theta \qquad (7)$$

$$\therefore S_I / S_R = tan\theta. \quad (8)$$

Based on Eqs. (5) and (8), τ is given by

$$\tau = tan\theta / \omega = S_I / \omega S_R. \quad (9)$$

Alternatively, based on Eqs. (6) and (7),

$$\sqrt{S_R^2 + S_I^2} = k_f N_0 \tau P_{E0}^2 / 2\sqrt{1 + (\tau\omega)^2} \propto k_f N_0 \tau P_{E0}. \quad (10)$$

Therefore, by calculating the S_I and the S_R, the fluorescence lifetime τ and the fluorescence intensity $k_f N_0 \tau P_{E0}$ can be determined using Eqs. (9) and (10), respectively (Fig. 3b and e). Finally, FRET efficiency (E) is given by

$$E = 1 - \tau_d' / \tau_d \quad (11)$$

where τ_d' and τ_d are the donor lifetimes in the presence and absence of the acceptor, respectively.

Here, we therefore introduce a microscopy-based protocol for evaluating drug responsiveness in leukemia cells from individual patients using FRET biosensors that we have developed and a protocol for the use of a unique instrument, the world's first flow cytometer designed to detect both the fluorescence lifetime and the fluorescence intensity. The fluorescence lifetime cytometer (FLiCM) enables high-throughput quantitative measurements of FRET efficiency in a single living cell, which might represent a suitable machine to improve the effectiveness and efficiency of present technologies.

2 Materials

Prepare all solutions using distilled deionized water (DDW, prepared by autoclaving deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents, unless otherwise specified. Diligently follow all waste disposal regulations when disposing of waste materials. Note that materials that are exposed to blood samples from patients should be treated as infectious waste.

2.1 The Isolation of Mononuclear Cells and Subsequent Cell Culture

1. Phosphate buffered saline (PBS): Dissolve 9.6 g of Dulbecco's PBS (−) in 1 L of DDW and autoclave at 120 °C for 15 min. Store at 4 °C until use.
2. 0.5 M ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) (pH 8.0) solution: Dissolve 93 g of EDTA, disodium salt, dehydrate [2NA(EDTA•2Na)] and 9 g of NaOH in approximately 400 mL of DDW. Adjust the pH to 8.0 using 10 N

NaOH and add DDW to a total of 500 mL, followed by sterilization using a 0.45 μm filter.

3. PBS buffer: Add 1/250 volume of 0.5 M EDTA (pH 8.0) solution to PBS. Store at 4 °C.
4. Lymphoprep.
5. 100 mM Sodium pyruvate solution (sterile-filtered), representing a 100× concentrated solution.
6. 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution: Dissolve 11.9 g of HEPES in approximately 40 mL of DDW. Adjust the pH to 7.2 using 10 N NaOH, add DDW to a total of 50 mL, and sterilize using a 0.45 μm filter.
7. RPMI 1640 working medium (complete RPMI-1640): Supplement RPMI-1640 medium with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum (FBS), 100× stabilized penicillin–streptomycin (P/S) solution (f.c. 100 U/mL and 0.1 mg/mL, respectively), and 1 mM sodium pyruvate. Store at 4 °C.
8. RPMI-1640 working medium without phenol red [complete RPMI-1640 phenol red (−)]: Supplement RPMI-1640 medium (no phenol red) with 10% FBS, 1 mM sodium pyruvate, P/S solution, and 15 mM HEPES. Store at 4 °C.
9. Red blood cell (RBC) lysis buffer (10 × conc.): Dissolve 82.6 g of NH_4Cl, 11.9 g of $NaHCO_3$, and 378 mg of $EDTA{\cdot}2Na{\cdot}2H_2O$ in DDW. Adjust the pH to 7.3 using concentrated HCl (35–37%), add DDW to a total of 1 L, and sterilize using a 0.45 μm filter.

2.2 Transfection

1. Transfection reagent: Nucleofector Kit V (Lonza, Basel, Switzerland). Add the entire Supplement (an adjuvant to transfection) to Nucleofector solution before use. Amaxa cuvettes are included in the kit.
2. Electroporator: Nucleofector II (Lonza).
3. Expression vector pPickles-2.31: *E. coli* JM109 strain was transformed with a plasmid harboring the coding sequence of the FRET-based biosensor Pickles [4] and then inoculated on a Lysogeny/Luria Broth (LB) agar plate containing 100 μg/mL ampicillin. The bacteria were cultured in 100 mL of LB media containing ampicillin, and the plasmids were purified using the JETSTAR 2.0 LFU Plasmid Midi Kit (GENOMED, Löhne, Germany) according to the manufacturer's instructions. At the final step of the preparation, recover the plasmid DNA in endotoxin-free water at a concentration of 3–5 μg/μl and store at −20 °C (*see* **Note 1**).

2.3 Drug Susceptibility Assessment

1. Tyrosine kinase inhibitors for BCR-ABL:
   Imatinib mesylate (IM) 10 mM in DDW.
   Nilotinib (NL): 10 mM in dimethyl sulfoxide (DMSO).
   Dasatinib: 100 μM in DMSO.
   Aliquot the drug solutions into 1.5 mL tubes (app. 50 μl each) and store at −20 °C.
2. Dish coating agent: 0.01 % poly-L-lysine solution.
3. EZVIEW 96-well Glass Bottom LB Culture Plates.

2.4 General Equipment

1. 24-well and 12-well cell culture plates.
2. Centrifuge: RL-101 swinging bucket rotor.
3. CO_2 incubator: CDI-165.

2.5 Equipment for Microscopic Observation (See Fig. 2)

1. Research inverted microscope: IX-81 (Olympus, Tokyo, Japan).
2. Objective lens: UPLSAPO 60XO, NA 1.35 (Olympus).
3. Light source: SORA SE light engine (Lumencor, Beaverton, OR, USA).
4. Cooled charge-coupled device (CCD) camera: CoolSNAP MYO (Photometrics, Tucson, AZ, USA).
5. Filter wheels and shutter control: MAC-5000 (Ludl Electronic Products, Hawthorne, NY, USA).
6. Stage incubator: WP-S-10E Chamlide (Live Cell Instrument, Seoul, Korea).
7. Motorized XY-stage: BIXY FFC (OL) (Chuo Precision Industrial, Tokyo, Japan).
8. Neutral density (ND) filter: 25 % (Olympus).
9. Dichroic mirror: XF2034 (455DRLP) (Omega Optical, Brattleboro, VT, USA).
10. Barrier filters: excitation filters XF1071 (440AF21 for CFP and FRET) and XF1068 (500AF25 for YFP); emission filters XF3075 (480AF30 for CFP) and XF3079 (535AF26 for FRET and YFP) (Omega Optical).
11. Software: MetaMorph Version 7.8 is used for the acquisition and manipulation of microscopic images, and Microsoft Excel Version 14.0 (2010) is for data analyses.

2.6 Flow Cytometry

1. FLiCM (Mitsui Engineering & Shipbuilding; Fig. 3a and Table 1).
2. Falcon™ Tube with Cell Strainer Cap.
3. 8.5 mL polystyrene FACS test tube (75 × 15.7 mm): (*see* **Note 2**).

Table 1
The specifications of the FLiCM

Feature	Specification
Light source	445 nm laser diode (60 mW)
Number of detection channels	
Scattering light	2 ch
Fluorescence intensity	3 ch
Fluorescence lifetime	3 ch
Detector	
Forward scatter	SiPD
Side scatter	PMT
Fluorescence intensity and lifetime	PMT
Detection (fluorescence) wavelength	
SSC	440 ± 5 nm
FL1	482 ± 17.5 nm
FL2	542 ± 13.5 nm
FL3	600 nm long pass
Sample flow rate	6 m/s
Sample flow volume (μl/min)	Lo: 40, Mid: 80, Hi: 160
Maximum acquisition rate	10,000 events/s
Modulation frequency	28 MHz
Size (mm)	500 (W) × 675 (D) × 700 (H)

SiPD silicon photodiode, *PMT* photomultiplier tube

4. Calibration beads: PeakFlow™ Blue or Cell Sorting Set-up beads (UV laser) (Life Technologies, Carlsbad, CA).
5. Data analysis software: FlowJo (Tommy Digital Biology Co., Ltd.).

3 Methods

Perform all procedures at room temperature and on a clean bench unless otherwise specified.

3.1 Mononuclear Cell Isolation from Bone Marrow Cells (BMCs)

1. Dilute BMCs in 1/7 volume of PBS buffer (i.e., BMC: PBS buffer = 7: 1) (*see* **Note 3**).
2. Dampen a cell strainer (100-μm pore size) with PBS buffer.

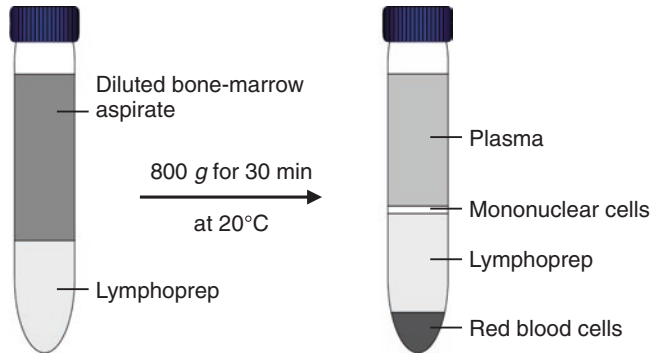

Fig. 4 Mononuclear cell isolation from BMCs. The *left panel* shows a sample tube filled with Lymphoprep and bone-marrow aspirates before centrifugation, and the *right panel* shows the sample after centrifugation

3. Pass the BMCs/PBS buffer through the filter into a fresh 50 mL centrifuge tube to remove the cell clumps and bone fragments.
4. Fill a 15 mL centrifuge tube with 3–4 mL of Lymphoprep (Fig. 4).
5. Carefully layer the filtrated BMCs/PBS buffer (app. 5 mL in each tube) above the Lymphoprep (*see* **Note 4**).
6. Centrifuge at 800 × *g* for 30 min at 20 °C (the brake must be disabled).
7. Recover the mononuclear cell layer fraction using a sterile Pasteur pipette (*see* Fig. 4).
8. Place the recovered aliquot in a 50-mL centrifuge tube; add complete RPMI-1640/PBS to a total volume of 50 mL, and mix by pipetting (*see* **Note 5**).
9. Centrifuge at 300 × *g* for 10 min to remove any remaining Lymphoprep.
10. If the pellet is colored red at this step, perform the following procedure (RBC lysis protocol) to remove the contaminating RBCs (**steps 11–16**) . Otherwise, skip ahead to **step 17**.
11. Dilute 10× conc. RBC lysis buffer to 1× conc. using cold endotoxin-free water (if the number of cells is less than 10^8, prepare 10 mL of 1× conc. RBC lysis buffer).
12. Discard the supernatant (after **step 9**) and suspend the pellet in 1× RBC lysis buffer.
13. Centrifuge at 300 × *g* for 5 min at 20 °C.
14. Discard the supernatant. Add cold PBS to a total of 15 mL and mix by pipetting.
15. Centrifuge at 300 × *g* for 5 min at 20 °C.
16. Repeat **steps 4** and **5**.

17. Discard the supernatant. Add complete RPMI-1640/PBS to a total of 50 mL, and centrifuge at 200 × *g* for 10 min to remove the platelets.
18. Discard the supernatant. Resuspend the cells in complete RPMI 1640, and transfer this suspension to a culture dish (*see* **Note 6**).
19. Place the culture dish in a CO_2 incubator at 37 °C, and culture for 1–4 h.

3.2 Transfection

1. Transfer the culture media containing 1×10^7 cells from a culture dish to a 15 mL centrifuge tube.
2. Centrifuge at 300 × *g* for 5 min. Then, discard all of the supernatant (*see* **Note 7**).
3. Add 100 μl of Solution V and 25 μg of Pickles 2.31 (*see* **Note 8**).
4. Mix gently, and transfer this suspension to an Amaxa cuvette.
5. Electroporate using the Nucleofector (Program T-020).
6. Add ~500 μl of pre-warmed medium using the Amaxa pipette and transfer the cells to a 90-mm dish prefilled with 10 mL of complete RPMI medium.
7. Culture these cells for 12–18 h at 37 °C.

3.3 Drug Treatment

1. Count the cells and transfer them to a 15 mL centrifuge tube.
2. Centrifuge at 300 × *g* for 5 min, and discard the supernatant.
3. Suspend the pellet in complete RPMI-1640 phenol red (−), and aliquot this cell suspension equally into four wells of either a 24-well or 12-well plate (*see* **Note 9**).
4. Add drugs to a final concentration of 2 μM for IM, 4 μM for NL, and 100 nM for DS (*see* **Note 10**).
5. Culture the cells for 24 h at 37 °C in a 5 % CO_2 atmosphere.

3.4 Fluorescence Microscopic Observation

1. Add 75 μl of poly-L-lysine per well to four wells of a 96-well glass bottom culture plate. Incubate for 1 h at 37 °C, and wash twice with 100 μl of PBS.
2. Turn on all devices associated with the microscope and the computer (*see* **Note 11**).
3. Transfer 1.0×10^5 cells to each well of the recoated 96-well plate (*see* **step 1**), and incubate for 10 min in a 5 % CO_2 incubator at 37 °C.
4. Open MetaMorph software.
5. Set the "Configure Illumination" for filter sets as follows (*see* **Note 12**):

   FRET: excitation filter, 440AF21; emission filter, 535AF26;

   CFP: excitation filter, 440AF21; emission filter, 480AF30; and

   YFP: excitation filter, 500AF25; emission filter, 535AF26.

6. Open "Multi Dimensional Acquisition," check "Multiple Wavelengths" in the "Main" tab, and select 4 for the "Number of Wavelengths" in the "Wavelength" tab. Set the exposure times for 4×4 binning to 200 and 50 ms for fluorescence imaging and differential interference contrast (DIC) imaging, respectively, in each configuration tab (*see* **Note 13**).
7. Acquire at least 100 cell images (of FRET, CFP, YFP, and DIC) per well/sample (*see* **Note 14**).

3.5 Microscopic Data Analysis

1. Open MetaMorph software.
2. Stack the images (*see* Fig. 5).

   Open the "Review Multidimensional Data" window. Select all files associated with the same sample, and select "Append Sets" to merge the files. Select the created merge file, and select "View". Check all image and wavelength boxes, and select "Load Images". Save the stacked images.
3. Subtract the background (Fig. 6).

   [Open the stack files of all fluorescence images (FRET, CFP and YFP). Create a background subtraction region (for instance, an area near the corner that is blank; *see* also **Note 15**) on the FRET image. Transfer this region to the CFP and YFP images using the "Transfer Regions" command. Open the

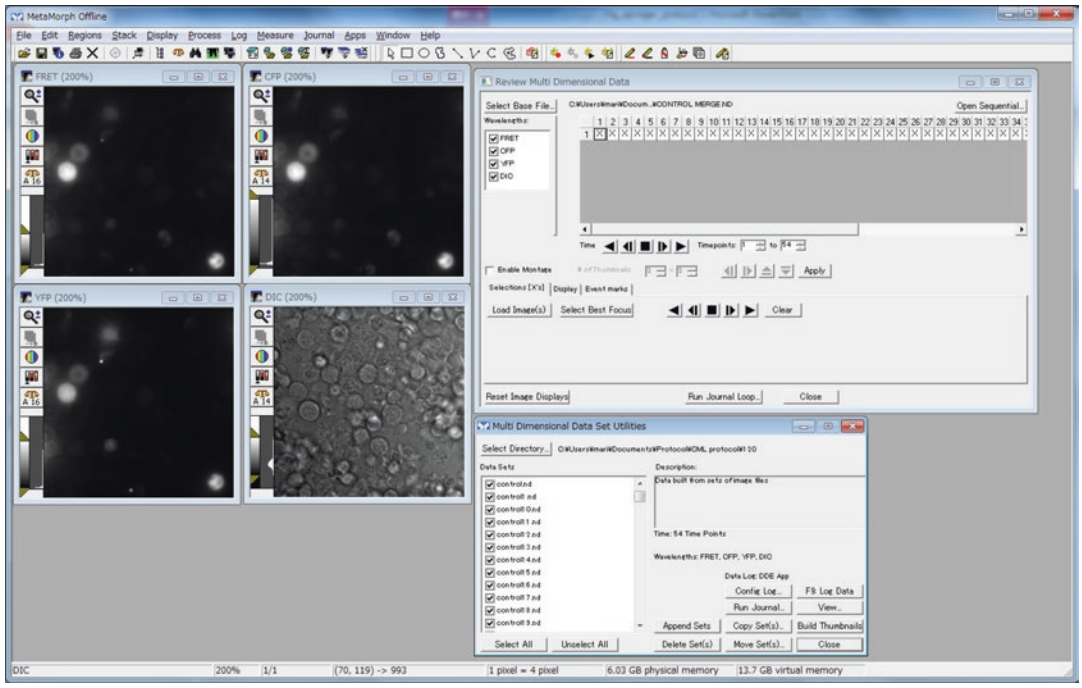

Fig. 5 Example of an application window during construction of a stack from image files. *See* Subheading 3.5 for detailed information

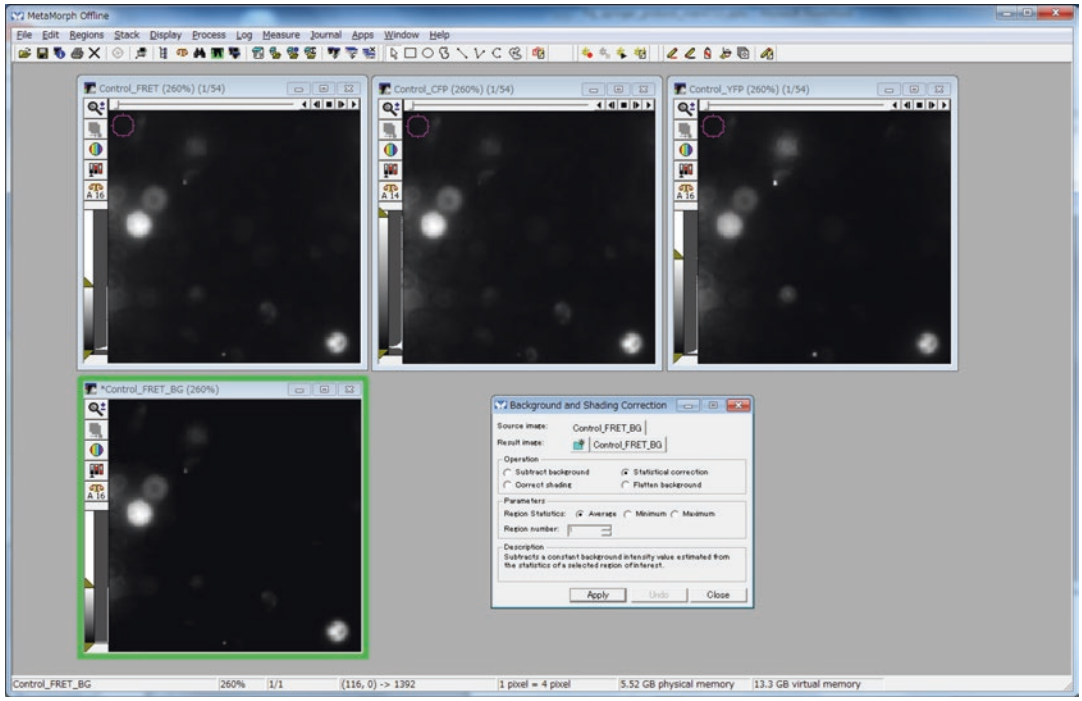

Fig. 6 Example of an application window during background subtraction. *See* Subheading 3.5 for detailed information

"Background and Shading Correction" window. Choose Souse (all planes) and Result Images, and check the "Operation: Statistical Correction" and "Parameters: Average" boxes. Select "Apply".]

Execute steps in parenthesis ([ ]) for both the CFP and YFP stack files. Save the resulting images.

4. Measure the fluorescence intensity (Fig. 7).

   Open the stack files of the background-subtracted fluorescence images (FRET, CFP, and YFP) and that of the DIC images. Create regions around each cell on the FRET image (*see* **Note 15**).

   [Open the "Region Measurement" window. Select "Data Log" to launch Microsoft Excel, and create an Excel file for data export. Choose the current plane of FRET as the source. In the "Configure" tab, confirm that the "Region Label", "Image Name", "Image Plane", "Area", "Average Intensity", "Intensity Standard Dev" boxes are checked. Select "F9: Log Data". Transfer the regions to the CFP and YFP images, and log the data in the same manner.]

   Execute steps in parentheses ([ ]) for all planes (*see* **Note 16**). Save the Excel file.

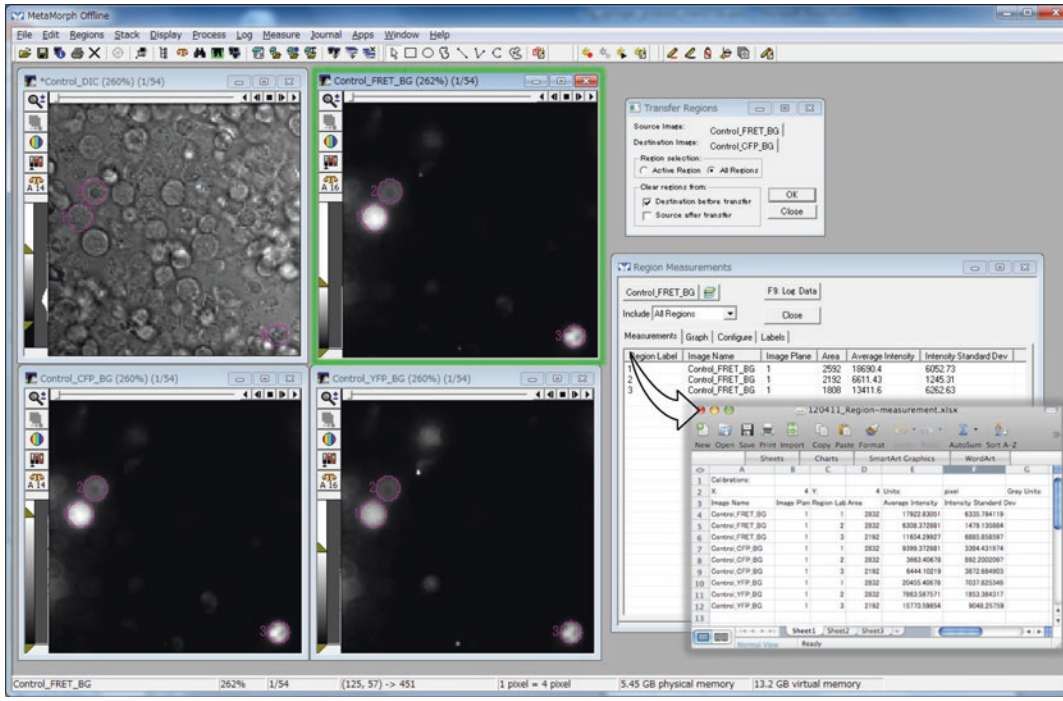

Fig. 7 Example of an application window during the measurement of fluorescence intensity and data logging. The *inset* on the *bottom right corner* shows a window of the Excel file to which log data are being transferred. *See* Subheading 3.5 for detailed information

5. Calculate the FRET efficiency.

   Open the Excel file. Sort the data in ascending order according to the Image Name. Place the Average Intensity and Intensity Standard Dev values for FRET and YFP next to those for CFP. Calculate the following values (*see* **Note 17**) (*see* Fig. 8):

   YFP_estimated = CFP_average intensity × 0.77;

   YFP_min = CFP_average intensity × 1.8665;

   YFP_max = CFP_average intensity × 3.1329;

   Emission ratio = FRET_average intensity/CFP_average intensity; and

   Emission ratio selected = IF (AND (YFP_average intensity > YFP_min, YFP_average intensity < YFP_max, CFP_average intensity > 100), emission _ ratio, " ").

   Create a scatter plot using the Emission ratio selected values (Fig. 9). The cells exhibiting an Emission ratio higher than D-FRET (=2.04) are estimated to be drug-resistant cells (*see* **Note 18**).

6. Determine the drug responsiveness.

   The drug responsiveness to each drug is determined based on the presence or absence of a decrease in the median value of

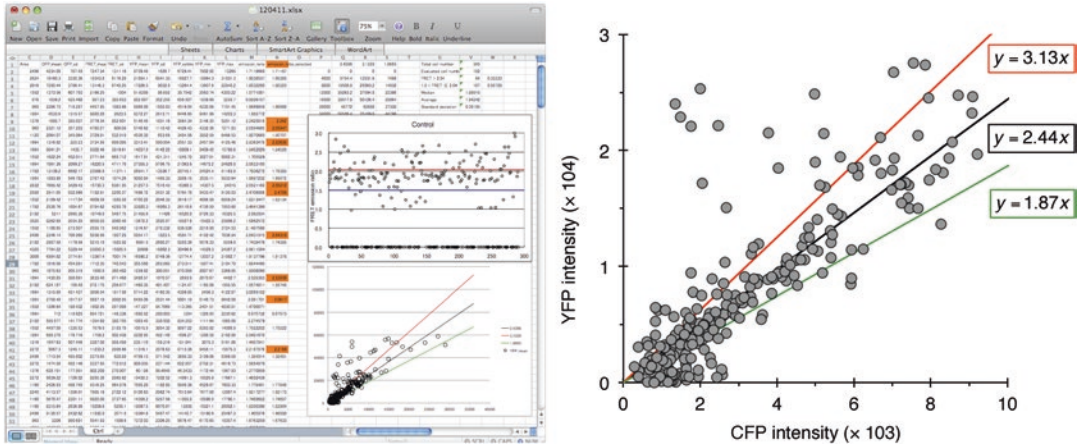

Fig. 8 Example of an application window during the calculation of FRET efficiency using Microsoft Excel. The *right panel* is the enlarged scatter graph within the *left panel* and is an *xy*-plot of the intensities of CFP and YFP in CML cells expressing Pickles. Because Pickles harbors a pair of fluorescent proteins, the intensities of CFP and YFP must display a linear correlation. Therefore, cells displaying intensity ratios of YFP/CFP between 1.87 and 3.13 (i.e., between the *red* and *green lines*) are subjected to analysis. The cells that do not satisfy these criteria (i.e., outside the *red* or *green line*) appear to express incomplete biosensors, which would not be expected to respond appropriately to drug treatment. Therefore, these cells are omitted from analysis

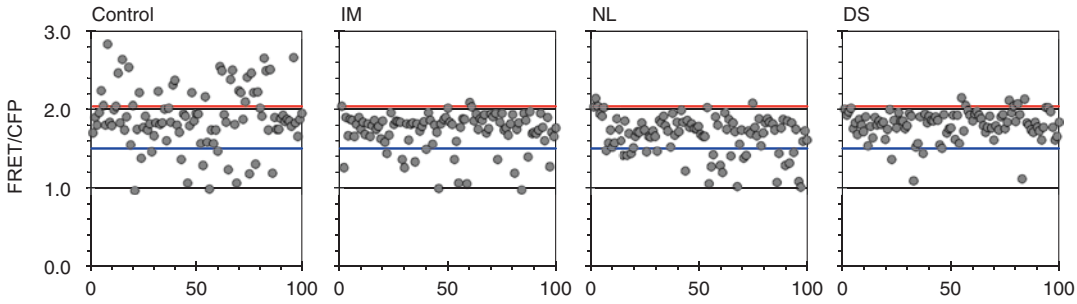

Fig. 9 Plot of the emission ratio (FRET) for all cells analyzed. The *abscissa* indicates the cell number, and the ordinate indicates the FRET efficiency of each cell. When the cells displaying a FRET efficiency greater than 2.04 remain after treatment, the patient is diagnosed as containing drug-resistant cells. Alternatively, the FRET efficiency of the overall cell population provides information about the drug responsiveness of the patient

the FRET efficiency. The presence of drug-resistant cells is ascertained based on the subset of cells displaying a FRET efficiency > 2.04 after drug treatment. In this case, because the FRET values tend to be decreased by all treatments, the patient is expected to respond to all of the drugs to some extent. However, the existence of cells displaying a high FRET efficiency suggests the development drug resistance during his/her therapeutic course.

3.6 Observation via Flow Cytometry

The protocols for mononuclear cell preparation, transfection, and drug treatment are identical to those for fluorescence microscopy. You can prepare these samples together and divide them at **step 3** in Subheading 3.4.

1. Turn on the FLiCM and the control/analyzing computer. Open the FLiCM data station (FDS), the control software for FLiCM, that is installed on this computer.
2. Prepare the flow cytometry system by applying air pressure to the sheath solution tank (*see* **Note 19**).
3. Clean the flow chamber.

   Remove a tube from the sample holder and turn on the 'Cleaning' button in the operation panel. 'Cleaning' mode, which washes the capillary flow channel and removes air bubbles, is completed in 16 s. Execute 'Cleaning' mode three times in a row. Place a test tube filled with FACS Flow in the sample holder.
4. Transfer 1.0×10^5 cells to a 15 mL tube. Centrifuge at $300 \times g$ for 5 min at room temperature, and discard the supernatant.
5. Add 1.5 mL of ice-cold PBS, and resuspend the cells by pipetting.
6. Pass the cells through the 35 μm-nylon filter incorporated into the tube cap of a Falcon™ Tube with Cell Strainer Cap and store on ice until use.
7. After briefly vortexing, transfer the cell suspension to the test tube optimized for FLiCM.
8. Before measurement, select and activate an appropriate format for the graph displayed in FDS. You can select time-waveform, histogram, and scattergram.
9. Place a test tube containing calibration beads diluted in FACS Flow in the sample holder, and push the start button.
10. Select the preview mode in FDS (which allows for measurement without saving). Measure the calibration beads, and verify the proper operation of the FLiCM by confirming that the median values of FSC, SSC, FL1 (fluorescence Ch1), and FL2 (fluorescence Ch2) in the histograms are within the prescribed ranges (FSC, 50–100 ch; SSC, 1000 ch~; FL1, 100 ch~; and FL2, 80 ch~) (*see* **Note 20**).
11. Transfer the cell suspension to a test tube, place the tube in the sample holder, and start the measurement by selecting the preview mode. Tune the voltage of the PMT and the attenuator so that the median values of FSC, SSC, FL1, and FL2 are within suitable ranges (FSC, 30–200 ch; SSC, 50–8000 ch; FL1, 5–2000 ch; and FL2, 10–8000 ch) (*see* **Note 21**).
12. Configure the number of cells to be measured (typically, we prefer to measure 100,000 cells), the folder for data saving,

and the file name in the measurement configuration dialog box in FDS.

13. Select the measure mode in FDS, which enables measurement with saving.
14. When the number of measured cells reaches the set value or the stop button is selected, the measurement is completed, and the acquired data are automatically saved.

3.7 Analysis of the Flow Cytometry Data

1. Open FlowJo software and create a new worksheet.
2. Open the FCS files of all data measured (control, IM, NL, and DS) using "Add Sample".
3. Display a scatter plot of the control sample (by double-clicking on the file name). Change the abscissa and the ordinate to FL1 (donor fluorescence intensity, 482 ± 17.5, CFP) and FL2 (acceptor fluorescence intensity, 542 ± 13.5, YFP), respectively. Create a polygon gate that includes cells expressing sufficient amounts of the biosensor (Fig. 10a, *see* **Note 22**).

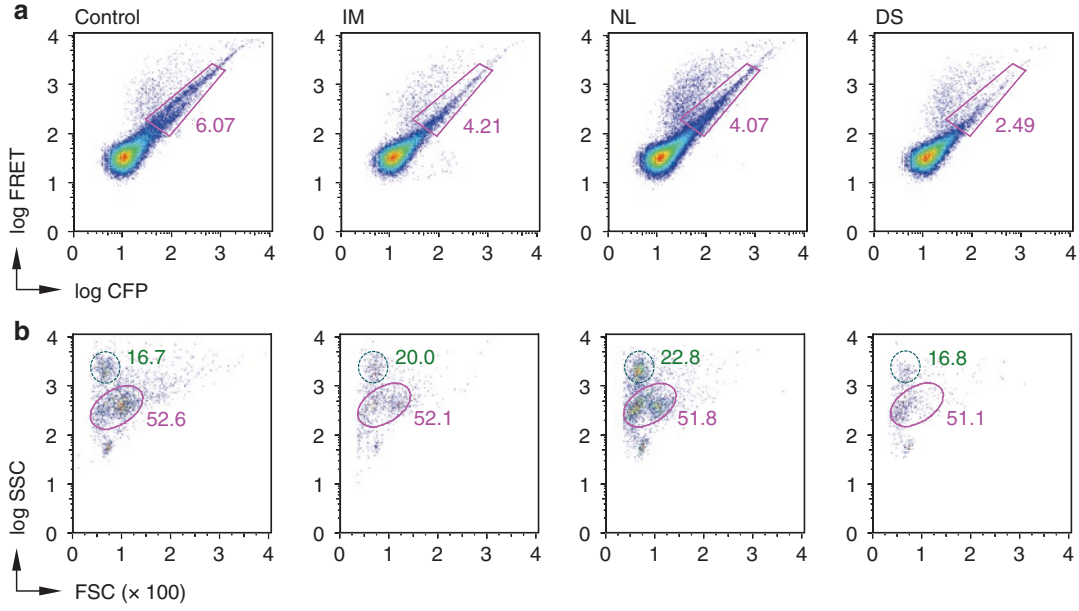

Fig. 10 Selection of cells appropriate for further analysis. (**a**) Selection of cells expressing the appropriate amount of the biosensor. CFP (FL1)-FRET (FL2) scattergrams are shown. All of the examined cells (Control and, IM, NL, or DS treatment) were plotted using an abscissa and an ordinate corresponding to the fluorescence intensity of CFP and FRET, respectively. A gating area was generated to satisfy the following criteria: the fluorescence intensity of both the donor and the acceptor is high; cells in which only either CFP or YFP is expressed are excluded. (**b**) FSC-SSC scattergrams for the cells selected in (**a**). Based on the FSC and SSC values, gating was performed to graphically distinguish between live (*magenta*, *solid circle*) and dead (*green*, *dashed circle*) cells, as shown

4. Select the region created in **step 3**, and open a new gate window displaying a scatter plot consisting of only the cells expressing a sufficient amount of the biosensor.
5. Change the parameters indicated for the abscissa and the ordinate to FSC and SSC, respectively.
6. Create two ellipsoidal gates that contain the live or dead cells (Fig. 10b, *see* **Note 23**).
7. Select either region generated in 6 to open a new window displaying a scatter plot consisting of the live or dead cells exhibiting optimal fluorescence intensities.
8. Select the dead cell subset and right-click on the file name of the workspace. Select "Derived Parameters," and define the emission ratio as FL2/FL1.
9. Change the parameters indicated for the abscissa and the ordinate to Tc1 [fluorescence lifetime of the donor (Tc1)] and Emission ratio. Confirm the location of the dead cells in the FL2/FL1-Tc1 scattergram (Fig. 11a, *see* **Note 24**).
10. Next, select the live cell subset, and create regions that include cells satisfying the following conditions (Fig. 11b) but that

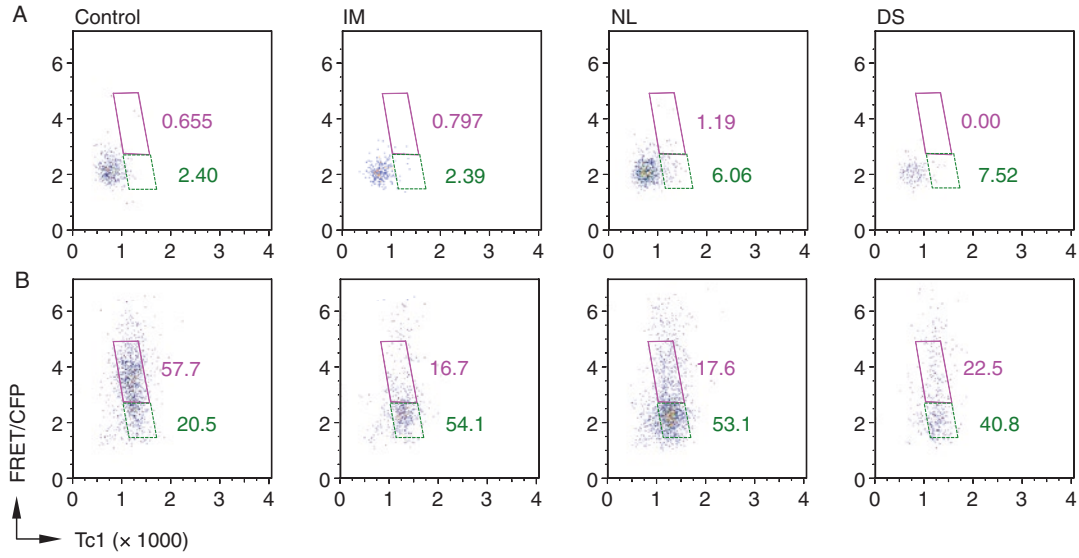

Fig. 11 Tc1-FRET/CFP scattergrams of dead cells (**a**) and live cells (**b**) displaying optimal expression levels of the biosensor. The scattergrams of dead cells are used to determine the gating area for the assessment of drug responsiveness. The regions of interest for the cells displaying $FRET^{high}$ and $FRET^{low}$ are set from the upper left to the lower right of the scatter plot. Because dead cells, which are determined based on their FSC and SSC values, are present in the lower left area, dead cells that cannot be distinguished on the basis of only the FSC-SSC profile might be included in this area, as shown in (**b**). Therefore, the aforementioned criteria are applied to exclude these dead cells

exclude the cells distributed in the lower left region (cells in this region are considered as dead; *see* also Fig. 11a and **Notes 22** and **25**):

(a) $2.8 \leq$ Emissi on ratio <5, $FRET^{high}$ population; and

(b) $1.5 <$ Emission ratio < 2.8, $FRET^{low}$ population.

11. Repeat **steps 3–10** for the files corresponding to the IM, NL, and DS samples.
12. Determination of drug responsiveness.

Any drug that exerts a 20% decrease in the percentage of $FRET^{high}$ cells is considered effective. When there remain cells in the region above Emission ratio = 5, it is possible that the patient will experience relapse and tolerance after treatment with such drug. We are currently accumulating data and evidence to define the diagnostic criteria for drug-resistance. In the present case, all of the drugs evaluated appear to be effective to some extent.

4 Notes

1. Dissolving DNA in TE buffer reduces the transfection efficiency, and contamination of endotoxins decreases the cell viability after electroporation; thus, the use of endotoxin-free water is highly recommended.
2. This tube is required because its inner diameter provides the optimal fit to the diameter of O ring of the sample holder (13 mm) of the FLiCM.
3. Because CML cells are concentrated in the mononuclear cell fraction, but not in the granulocyte fraction, we utilize this protocol in order to isolate leukemia cells.
4. Lymphoprep:diluted BMC should be approximately 3:7.
5. Because the pellet of the leukemia cells tends to agglomerate, we recommend two-step resuspension: e.g., first, add 5 mL of medium (or solution) to the pellet using a 5 mL pipette and mix well by pipetting, and then add the remaining solution and mix again.
6. When the number of cells is less than 10^7, resuspend the cells in 5 mL of RPMI and transfer this suspension to a 60-mm dish. When the cell number exceeds 10^7, use 10 mL of RPMI and a 90-mm dish.
7. Because FBS interferes with electroporation and decreases the transfection efficacy, you should remove the media so that the residual FBS is as little as possible.
8. If the number of cells is less than 1×10^7, proportionally decrease the amount of the plasmid. However, do not change the amount of Solution V.

9. When the number of cells is more than 10^6, resuspend the cells in 8 mL of RPMI and transfer 2 mL to each well of a 12-well plate. When the number of cells is less than 10^6, resuspend cells in 4 mL of RPMI and transfer 1 mL to each well of a 24-well plate.
10. IM and NL should be diluted 1/10 using DDW and then added to the wells. Precipitates will appear if they are directly added to the medium.
11. For reduction of drift induced by temperature changes, we recommend turning on the devices at least 2 h prior to observation.
12. Once you save these configurations, you can skip this step.
13. Although the optimal exposure times may vary depending on the microscope settings, you should keep them proportional to those which have been predetermined so that the data across experiments can be compared. Particular attention should be paid avoiding saturation of the fluorescence intensity. The exposure times of ECFP and FRET should be identical.
14. Leave one corner of the images blank to make it easier to subtract the background.
15. Exclude any dead cells (which can be estimated using the DIC images) and any cells in which the biosensors are eccentrically localized in the image.
16. We made Journals (a sort of macro in MetaMorph software) to semi-automatically perform image data analysis.
17. These coefficients may vary depending on the microscope settings and were determined using 293F cells in our case. These calculations are required to eliminate the cells in which the Pickles biosensors are cleaved. Cleaved biosensors are not expected to display an appropriate response to drug treatment.
18. The D-FRET value is defined as the mean FRET efficiency + 3 × S.D. in 293F cells, which are expected to lack BCR-ABL [4].
19. The reagents required to maintain FLiCM are identical to those used for standard flow cytometers: e.g., Sheath fluid, BD FACS Flow Sheath Fluid (BD Biosciences); Clean liquid, BD FACS Rinse Solution (BD Biosciences); Disinfecting clean liquid, BD FACS Clean Solution (BD Biosciences).
20. When the measurement begins, the sample is extruded using the pressure of the sheath fluid, which generates a laminar flow of cells at the center of the flow cell. The laser beam is focused using two lenses to irradiate the cells passing through the flow cell. The irradiated cells scatter the laser beam and simultaneously emanate fluorescence from the fluorophore expressed in the cells. Forward-scattered light (FSC) is detected using a silicon photodiode (SiPD). The intensity of FSC can be used not

only as an indicator of cell size but also as a trigger generator for the fluorescence lifetime and intensity measurement by regarding the input of the FSC signal as the presence of the cells at the laser spot. Given that the size of the cells varies in a context-dependent manner, the gain and the threshold level for triggering should be appropriately adjusted to obtain optimal values for each cell type. Alternatively, side-scattered light (SSC) and fluorescence are collected using condenser lenses, which are subsequently separated into the desired wavelength regions using dichromatic and band-pass filters and are individually detected using photomultiplier tubes (PMTs).

21. The photons detected by the PMTs are internally transformed into electrical signals. The amplitude of the output signal is proportional to the fluorescence intensity ($k_f N_0 \tau P_{E0}$) but varies by more than one order of magnitude depending on the fluorescent dye used. Therefore, the control voltage of the attenuator should be adjusted to obtain the appropriate output value. The output signal of the PMT undergoes amplification and demodulation in the signal processing circuit, and the resulting data, including scatted light (2 ch), fluorescence intensity (3 ch), and fluorescence lifetime (3 ch), for every event are displayed and stored in the analyzing PC.
22. Ensure that the created gate does NOT include cells expressing only CFP or YFP and cells displaying fluorescence intensity of either the donor or the acceptor is saturated.
23. In general, FSC represents the cell size, whereas SSC represents the complexity of the intracellular architecture (e.g., nuclear and organellar morphology and membrane structure). Therefore, FSC intensity of dead cells is lower than that of living cells because of their reduced size. By contrast, SSC intensity of dead cells becomes greater than that of living cells owing to the membrane collapse of dead cells. According to this information, gates can be created to distinguish live cells from dead cells in most established cell lines, which may be performed at the first step of cytometric data analysis. However, in the case that primary cells are utilized, FSC-SSC scattergrams do not always display a typical pattern, and living cells may not be well demarcated in the scattergram. Dead cells that cannot be distinguished in the scattergram can be discriminated using the Tc1-ratio scattergram, as described below, demonstrating the usefulness of this method.
24. In the presence of FRET, the emission ratio and the fluorescence lifetime are increased and decreased, respectively, and vice versa. Therefore, the populations of dead cells in the lower left region that cannot be distinguished based on their FSC-SSC profile but should be excluded from further analysis

remain in Fig. 11b. Notably, these cells cannot be distinguished based on only their emission ratio or fluorescence lifetime, despite their substantial impact on the final results, demonstrating the benefit of the use of the FLiCM to measure both parameters.

25. Because the FRET efficiency represents BCR-ABL activity, a decrease in the percentage of $FRET^{high}$ cells after drug treatment is implicated in the effectiveness of the drug to suppress BCR-ABL activity in the entire cell population. Alternatively, the presence of cells that remain in the region > $FRET^{high}$ indicate that these cells might display resistance to the relevant drug.

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