Please cite this article as: Futamata *et. al.*, (2021). Live Cell FRET Analysis of the Conformational Changes of Human P-glycoprotein,*Bio-protocol* 11 (4): e3930. DOI: 10.21769/BioProtoc.3930.

bio-protocol www.bio-protocol.org/e3930 Bio-protocol 11(04): e3930. DOI:10.21769/BioProtoc.3930

Live Cell FRET Analysis of the Conformational Changes of Human P-glycoprotein

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[Abstract] The molecular mechanisms of P-glycoprotein (P-gp; also known as MDR1 or ABCB1) have been mainly investigated using artificial membranes such as lipid-detergent mixed micelles, artificial lipid bilayers, and membrane vesicles derived from cultured cells. Although these *in vitro* experiments help illustrate details about the molecular mechanisms of P-gp, they do not reflect physiological membrane environments in terms of lateral pressure, curvature, constituent lipid species, *etc.* The protocol presented here includes a detailed guide for analyzing the conformational change of human P-gp in living HEK293 cells by using intramolecular fluorescence resonance energy transfer (FRET), in which excitation of the donor fluorophore is transferred to the acceptor without emission of a photon when two fluorescent proteins are in close proximity. Combining FRET analysis with membrane permeabilization, the contribution of small molecules such as nucleotides to the conformational change can be evaluated in living cells.

Keywords: Live cell analysis, Intramolecular FRET, Conformational change, P-glycoprotein, ABC transporter, Membrane protein

[Background] P-glycoprotein (P-gp) is an ATP-driven multidrug transporter that extrudes various hydrophobic toxic compounds to the extracellular space. P-gp consists of two transmembrane domains (TMDs) that form the substrate translocation pathway and two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP. At least two P-gp states are required for the transport. In the inward-facing (pre-drug transport) conformation, the two NBDs are separated, and the two TMDs are open to the intracellular side; in the outward-facing (post-drug transport) conformation, the NBDs are dimerized, and the TMDs are slightly open to the extracellular side (Kodan *et al.*, 2020). Since the discovery of P-gp (Juliano and Ling, 1976; Chen *et al.*, 1986; Ueda *et al.*, 1986), numerous studies have been conducted to clarify its transport mechanism. Most have utilized artificial membrane environments such as lipid-detergent mixed micelles (Kodan *et al.*, 2014 and 2019; Verhalen *et al.*, 2017), artificial lipid bilayers (Verhalen *et al.*, 2012; Moeller *et al.*, 2015; Zoghbi *et al.*, 2017; Dastvan *et al.*, 2019), or membrane vesicles derived from cells overexpressing P-gp (Liu and Sharom, 1996; Qu and Sharom, 2001). However, these conditions do not reflect physiological membrane environments in terms of lateral pressure, curvature, or constituent lipid species. Because these environmental factors can affect the function of P-gp, the detailed transport mechanism should be investigated in living cells. Accordingly, a conformation-sensitive monoclonal antibody, UIC2, has been used (Bársony *et al.*, 2016). However, because UIC2 binds to the extracellular loops of the inward-facing structure of P-gp, the conformational

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change of the intracellular side has not been revealed. The distances between the C termini of NBD1 and NBD2 are estimated to be about 30 and 11 Å in the inward-facing and outward-facing structures, respectively (Futamata *et al.*, 2020) and this difference in distance is assumed to be one of the largest between the inward-facing and outward-facing structures. Therefore, we monitored the distance of the two NBDs by FRET analysis. When the donor and acceptor were attached to different domains of the macromolecule, strong FRET occurred when the two domains were in close proximity. The big advantage of FRET analysis is that it can be performed in living cells in real time, which is not true of antibody-based analyses. In this study, two fluorescent proteins, monomeric (m)Cerulean and monomeric (m)Venus, were fused to the N- and C-terminal regions of NBDs, respectively. A change in distance between the two NBDs was evaluated by investigating the sensitized-emission of mVenus (acceptor) elicited during the excitation of mCerulean (donor) in living cells in real time. While the protocol described focuses on human P-gp in living HEK293 cells, it is also applicable to other membrane proteins.

Materials and Reagents

1. 35 mm glass-based dish (IWAKI, catalog number: 3971-035)
2. 100 mm cell culture dish (Falcon, catalog number: 353003)
3. 1.5 ml tube (Watson, catalog number: 131-815C)
4. 15 ml tube (Corning, catalog number: CLS430791)
5. 0.22 μm filter (Millipore, catalog number: SLGP033RS)
6. Mammalian expression vector that encodes FRET probe (see the beginning of the section Procedure below)
7. HEK293 cells (ACTT® CRL-1573™)
8. Poly-L-lysine (PLL) solution 0.01% (Sigma-Aldrich, catalog number: P4707-50ML)
9. DMEM (Nacalai Tesque, catalog number: 08458-16)
10. 0.5% Trypsin-EDTA (10×) (Gibco, catalog number: 15400-054)
11. Fetal Bovine Serum (Gibco, catalog number: 10270-106)
12. Lipofectamine LTX with PLUS Reagent (Thermo Fisher Scientific, catalog number: 15338100)
13. Opti-MEM (1×) (Gibco, catalog number: 2151680)
14. FluoroBrite DMEM (Gibco, catalog number: A1896701)
15. Sodium pyruvate (100 mM) (Gibco, catalog number: 11360-070)
16. GlutaMAX (100×) (Gibco, catalog number: 35050061)
17. Verapamil chloride (Wako, catalog number: 228-00783)
18. PEI MAX (MW = 40,000) (Polysciences, catalog number: 24765-1)
19. Adenosine 5’-triphosphate (ATP) disodium salt (Oriental Yeast, catalog number: 45142000)
20. Streptolysin O (SLO) (BioAcademia, catalog number: 01-531)
21. DAPI (Sigma-Aldrich, catalog number: D9542-1MG)
22. NaCl (Nacalai Tesque, catalog number: 31320-05)

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23. $Na_2HPO_4 \cdot 12H_2O$ (Nacalai Tesque, catalog number: 31723-35)
24. KCl (Nacalai Tesque, catalog number: 28514-75)
25. KH_2PO_4 (Nacalai Tesque, catalog number: 28721-55)
26. HEPES (Nacalai Tesque, catalog number: 17514-15)
27. KOH (Nacalai Tesque, catalog number: 28616-45)
28. CH_3COOK (Wako catalog number: 160-03175)
29. $MgCl_2 \cdot 6H_2O$ (Nacalai Tesque, catalog number: 20909-55)
30. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2650)
31. NaOH (Nacalai Tesque, catalog number: 31511-05)
32. PLL solution (see Recipes)
33. 10× PBS(-) (see Recipes)
34. 1× PBS(-) (see Recipes)
35. 10× transport buffer (see Recipes)
36. 1× transport buffer (see Recipes)
37. 0.05% Trypsin-EDTA (see Recipes)
38. 100 mM ATP stock solution (see Recipes)
39. 50 mM verapamil stock solution (see Recipes)
40. 1 mg/ml DAPI stock solution (see Recipes)
41. 100 mM NaN_3 stock solution (see Recipes)
42. 1 M $MgCl_2$ stock solution (see Recipes)
43. 1 mg/ml PEI-MAX (see Recipes)

Equipment

1. Autoclave (TOMY, model: LSX-500)
2. Vortex mixer (Scientific Industries, model: VORTEX-GENIE 2)
3. Temperature bath (TAITEC, model: SDminiN)
4. CO_2 incubator (Thermo Fisher Scientific, model: Forma 310 Direct Heat CO_2 incubator)
5. Centrifuge (TOMY, model: LC-200, rotor: TS-7LB)
6. Cell counter (DeNovix, model: Cell Drop BF)
7. Confocal laser scanning microscope (Carl-Zeiss, model: LSM700) operated by Zen 2012 and equipped with an objective lens (Plan-Apochromato ×63/1.4 NA oil immersion), an incubation chamber, temperature module S, and CO_2 module S.

Software

1. ImageJ-based Fiji (version 1.52p) (https://imagej.net/Fiji) (Schindelin *et al.*, 2012)
2. FRET and Colocalization Analyzer (https://imagej.nih.gov/ij/plugins/fret-analyzer/fret-analyzer.htm) (Hachet-Haas *et al.*, 2006)

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Procedure

For this protocol, we used human P-gp FRET probes (Futamata *et al.*, 2020). Schematic representations of the fluorescent protein-tagged P-gps are shown in Figure 1. Human P-gp FRET probes are available from the corresponding author, Kazumitsu Ueda (ueda.kazumitsu.7w@kyoto-u.ac.jp).

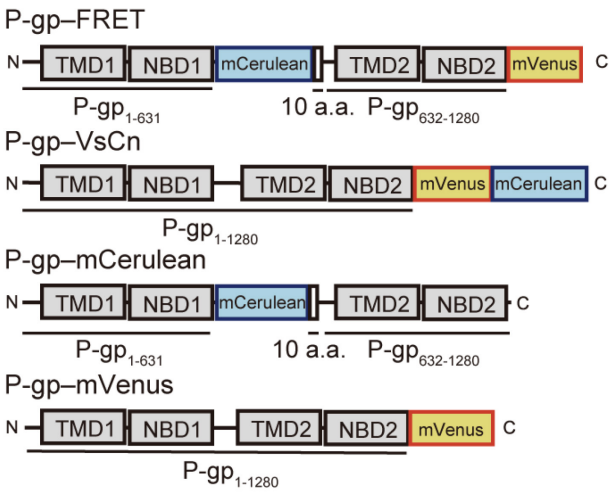

Figure 1. Schematic representations of fluorescent protein-tagged P-gps. We generated a FRET construct, human P-gp–FRET, in which mCerulean (donor) was inserted after NBD1 and mVenus (acceptor) was fused after NBD2. Another FRET construct, P-gp–VsCn, in which mVenus and mCerulean were fused tandemly after NBD2, was predicted to show a high level of FRET despite the conformation. P-gp–mCerulean, in which mCerulean was inserted after NBD1, and P-gp–mVenus, in which mVenus was fused after NBD2, were constructed as negative controls. TMD, trans-membrane domain. NBD, nucleotide-binding domain, a.a., amino acid.

Note: HEK293 cells are maintained in DMEM supplemented with 10% FBS (10%FBS-DMEM) in a 100 mm cell culture dish at 37 °C under 5% CO_2. For incubation, a CO_2 incubator was used.

A. Intact cells
   1. Transient expression of fluorescent protein-tagged P-gps
      a. **Day 1** Add 100 µl PLL solution to the 35 mm glass-based dish and incubate for 30 min at room temperature.
         Note: PLL solution was used to promote cell adhesion to the dish.
      b. Wash with 1.5 ml 1× PBS(-) twice.
      c. Prepare HEK293 cells cultured in the 100 mm cell culture dish and discard the culture medium.
      d. Wash with 1.5 ml 1× PBS(-) twice
      e. Add 1 ml 0.05% EDTA-Trypsin and incubate for 2 min at 37 °C under 5% CO_2.
      f. Add 9 ml 10% FBS-DMEM and transfer to a 15 ml tube.

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g. Centrifuge at 1,100 × *g* for 2 min at room temperature.
h. Discard the supernatant and add 10 ml 10% FBS-DMEM.
i. Count the number of cells using CellDrop following the manufacturer's protocol.
j. Dilute the cells to 1.5 × 10^5 cells/ml.
k. Seed HEK293 cells to the glass-based dish at 3.0 × 10^5 cells/well and incubate for 24 h at 37 °C under 5% CO_2.
l. **Day 2** Prepare the DNA-lipid complex.
Note: The minimum plasmid DNA set is P-gp–FRET, two negative controls (P-gp–mCerulean and P-gp–mVenus), and one positive control (P-gp–VsCn).
    i. Mix 2.5 µg plasmid DNA and 2.5 µl PLUS Reagent in 500 µl Opti-MEM and mix with the vortex mixer.
    ii. Incubate for 5 min at room temperature.
    iii. Add 6.25 µl Lipofectamine LTX and mix with the vortex mixer.
    iv. Incubate for 30 min at room temperature.
m. Replace the medium with 500 µl DNA-lipid complex and 1.5 ml 10% FBS-DMEM and incubate for 23 h at 37 °C under 5% CO_2.

2. Image acquisition
a. **Day 3** Wash with 2 ml FluoroBrite DMEM twice.
b. Replace the medium with 1 ml FluoroBrite DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 1× GlutaMAX and incubate for 1 h.
c. Transfer the dish to the incubation chamber equipped to the microscope.
d. Transfer 500 µl medium to a 1.5 ml tube and mix with 2 µl verapamil chloride stock solution (final 100 µM).
Notes:
    i. Do not add verapamil chloride stock solution to the glass-based dish directly because HEK293 cells can be peeled off by pipetting.
    ii. Verapamil is a P-gp substrate.
e. Return the medium to the glass-based dish and further incubate for 5 min.
f. Acquire images of the mVenus, mCerulean, and FRET signals according to the parameters below and save as .lsm or .czi format.
mVenus is excited at 488 nm, and the fluorescence emission is collected using a band pass filter (521-600 nm) (Figure 2). mCerulean is excited at 445 nm, and the fluorescence emission is collected using a short-pass filter (490 nm). For the FRET signal image, the laser is set to 445 nm, and the sensitized emission is collected using a band pass filter (521-600 nm) (Figure 3).
Note: Representative raw images of every channel of all four plasmids are shown in Figure 4.

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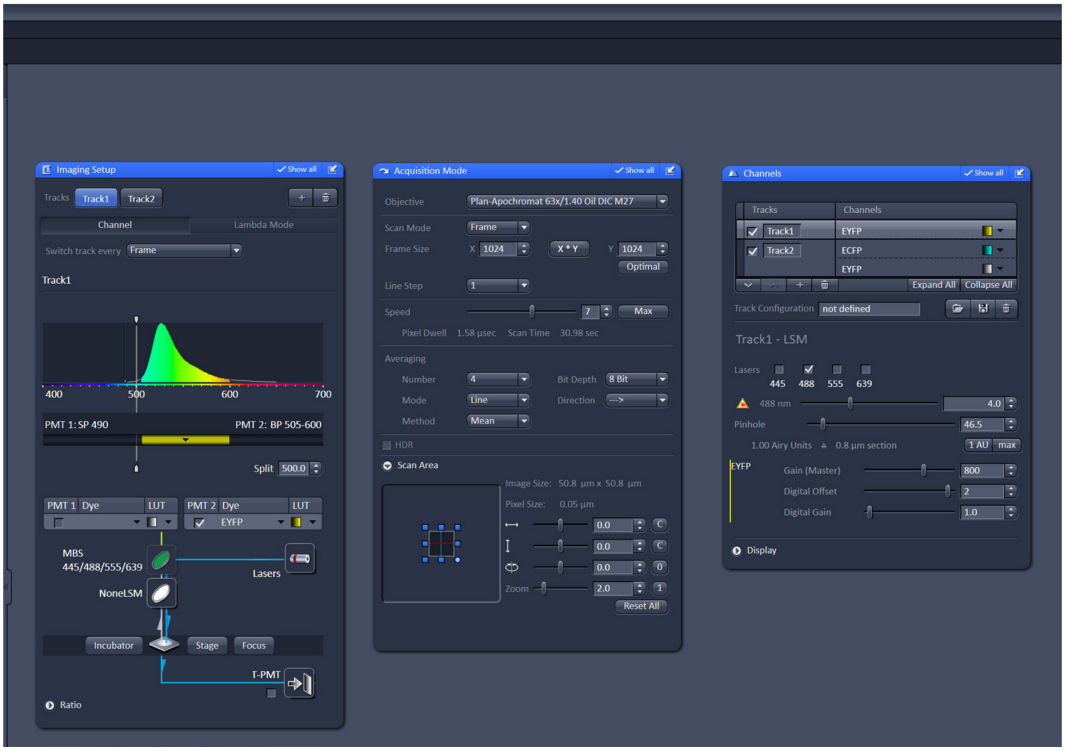

Figure 2. Zen software settings for mVenus imaging

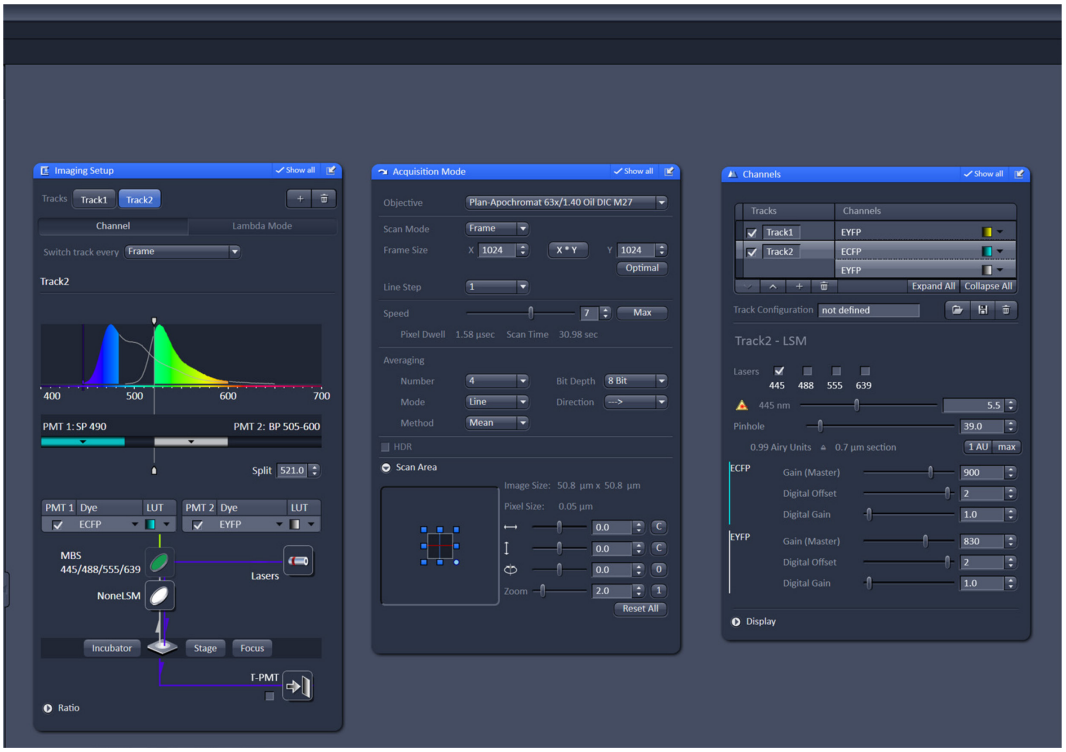

Figure 3. Zen software settings for mCerulean imaging and FRET signal imaging

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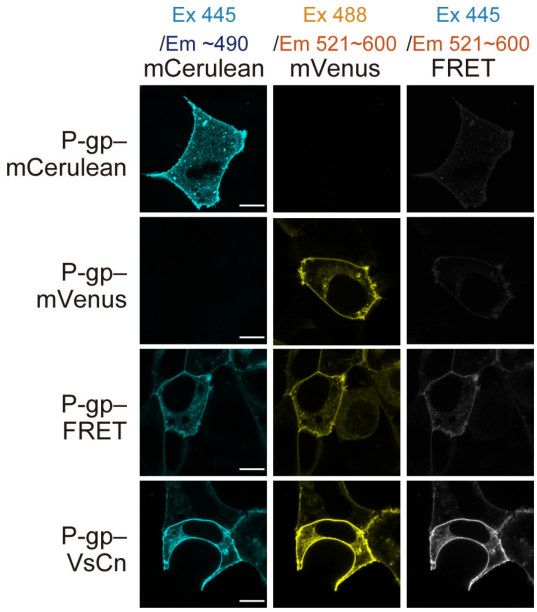

Figure 4. Representative raw images of every channel for all four plasmids. Scale bars = 10 μm.

B. Semi-intact cells

Note: Membrane permeabilization is suitable for manipulating the concentration of small molecules such as nucleotides.

1. Transient expression of the fluorescent protein tagged P-gp constructs.
   a. **Day 1** Seed the HEK293 cells as described in Steps A1a-A1k.
   b. **Day 2** Prepare the DNA-lipid complex as below.

      Note: Do not use Lipofectamine LTX when performing membrane permeabilization because it inhibits the pore formation by SLO.

      i. Mix 2 μg plasmid DNA and 98 μl Opti-MEM in a 1.5 ml tube and mix with the vortex mixer.
      ii. Mix 10 μl 1 mg/ml PEI-MAX and 90 μl Opti-MEM in another 1.5 ml tube and mix with the vortex mixer.
      iii. Mix the DNA and PEI-MAX and mix with the vortex mixer.
      iv. Incubate at room temperature for 30 min.
   c. Replace the medium with 200 μl DNA-PEI complex and 1.8 ml 10% FBS-DMEM and incubate for 24 h at 37 °C under 5% CO_2.
2. Membrane permeabilization and image acquisition

   Note: We recommend performing this step using one or two samples at a time in order to avoid the cells permeabilizing for a long time.

   a. **Day 3** Wash with 2 ml ice-cold 1× PBS(-).
   b. Add 1 ml serum-free DMEM containing 50 ng/μl SLO and incubate for 5 min on ice.

      Notes:

      i. Dilute SLO just before use.

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ii. SLO is a streptococcal toxin which forms 25-30 nm aqueous pores within the plasma membrane, which allows the free passage of ions and small molecules.

c. Wash with 2 ml ice-cold 1× PBS(-) three times.
d. Add 1 ml 1× transport buffer pre-heated at 37 °C supplemented with 2 mM $MgCl_2$, 5 mM ATP disodium salt, and 2 μg/ml DAPI. For ATP depletion, add 1× transport buffer supplemented with 2 mM $MgCl_2$, 2 μg/ml DAPI, and 10 mM NaN_3.
e. Incubate for 10 min at 37 °C under 5% CO_2.
f. Wash twice with 2 ml 1× transport buffer pre-heated at 37 °C.
g. Add 1 ml of the pre-heated 1× transport buffer supplemented with 2 mM $MgCl_2$, 5 mM ATP disodium salt, and 100 μM verapamil chloride.
h. Transfer the dish to the incubation chamber equipped to the microscope and incubate for 5 min at 37 °C under 5% CO_2.
i. Acquire images of the mVenus, mCerulean, and FRET signals of DAPI-positive cells according to the parameters described in Step A2f.

Data analysis

The FRET efficiency (proximity ratio) was calculated using Equation 1.

$$Proximity\ ratio = \frac{I_{DA} - aI_{AA} - dI_{DD}}{I_{DD}} \text{ (Eq. 1),}$$

where I_{DA} is the FRET signal or the sensitized emission of the acceptor during donor excitation (excitation 445 nm/emission 521-600 nm), I_{DD} is the donor fluorescence during donor excitation (excitation 445 nm/emission 490 nm), and I_{AA} is the acceptor fluorescence during acceptor excitation (excitation 488 nm/emission 521-600 nm). a and d are the bleed-through of the acceptor and donor, respectively.

Segmentation of the plasma membrane and calculation of the proximity ratio:

1. Open the .lsm or .czi format images with ImageJ and convert to .tiff format.
2. Split the image to each channel (Image menu > Color > Split Channels).
3. Calculate the donor and acceptor bleed-through from the images expressing P-gp–mCerulean and P-gp–mVenus, respectively, using “FRET and Colocalization Analyzer”.
   a. Download “FRET and Colocalization Analyzer” from https://imagej.nih.gov/ij/plugins/fret-analyzer/fret-analyzer.htm to the plugins folder.
   b. Restart ImageJ to add the "Fret Analyzer" command to the Plugins menu.
   c. Open “FRET Analyzer” from the Plugins menu.
   d. For the donor bleed-through evaluation, input the number of controls (up to 10 controls).
   e. Select the donor channel image and the FRET signal image of the first control.
   f. Click the “Get” button.

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g. Perform (c) and (d) for all controls and acquire the mean of the donor bleed-through.
h. Calculate the acceptor bleed-through the same way as the donor bleed-through.

4. If necessary, crop the images so that only a single cell is visible.
5. Save the images of the mCerulean, mVenus, FRET signals to separate folders.
6. Subtract the donor and acceptor bleed-through from the FRET signal image and create a corrected FRET (cFRET) image. This procedure is automatically performed by the home-made ImageJ macro described below.

Notes:

a. To use the following ImageJ macro, save the macro text as .txt format in the Macro subfolder in the ImageJ directory. Install the macro (Plugins > Macros > Install). Run the macro (Plugins > Macros > macro name).

b. Before executing this ImageJ macro, uncheck "Scale when converting" (Edit menu > Options > Conversions…).

```
setBatchMode(true); //Batchmode ON
// setBatchMode(false); //Batchmode OFF

dir1 = getDirectory("Select mCerulean folder");
//Select the folder in which the mCerulean images are saved
dir2 = getDirectory("Select mVenus folder");
//Select the folder in which the mVenus images are saved
dir3 = getDirectory("Select FRET signal folder");
//Select the folder in which the FRET images are saved
dir4 = getDirectory("Select cFRET folder");
//Select the folder in which the cFRET images are to be saved

list1 = getFileList(dir1);
list2 = getFileList(dir2);
list3 = getFileList(dir3);

A = getNumber("Enter donor BT",0);
B = getNumber("Enter acceptor BT",0);

for (i = 0; i < list1.length; i++){
open(dir1 + list1[i]);
c = getTitle();
run("32-bit");
//convert 8 bit image to 32 bit image
run("Duplicate...", "title=mc");
run("Select All");
```

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```
setColor(A);
fill();
imageCalculator("Multiply", "mc", c);
open(dir2 + list2[i]);
v = getTitle();
run("32-bit");
run("Duplicate...", "title=mv");
run("Select All");
setColor(B);
fill();
imageCalculator("Multiply", "mv", v);
open(dir3 + list3[i]);
f = getTitle();
run("32-bit");
imageCalculator("Subtract", f, "mc");
imageCalculator("Subtract", f, "mv");
run("8-bit");
retstr = split(f,"_");
Save_name = dir4 + retstr[0] + "_cF";
saveAs("Tiff", Save_name);
run("Close All");
}
```

7. Segment the plasma membrane and evaluate the proximity ratio on the plasma membrane. This procedure is automatically performed by the home-made ImageJ macro described below.
   Notes:
   a. *Representative images of the segmented plasma membrane are shown in Figure 5.*
   b. *Representative results of quantification of the proximity ratio are shown in Figure 6.*

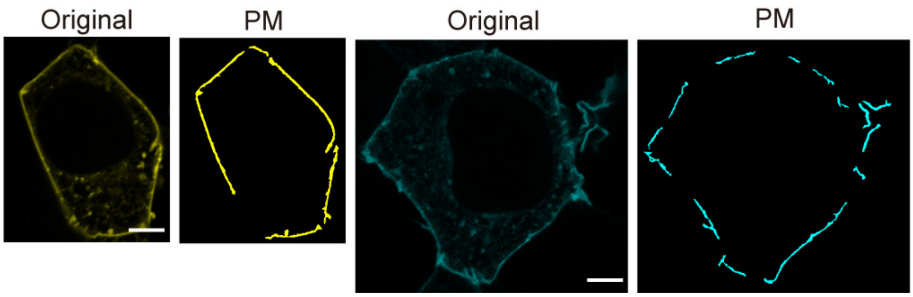

Figure 5. Representative images of the original membrane and segmented plasma membrane. Scale bars = 5 μm.

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P-gp-mCerulean		
Total ratio	Area	Average ratio
230.1832	2452	0.0939
154.5441	1853	0.0834
113.736	1316	0.0864
213.679	2540	0.0841
305.4575	3437	0.0889
158.4013	1719	0.0921
295.38	3207	0.0921
254.1358	3155	0.0806
144.8516	1474	0.0983
90.6951	969	0.0936

P-gp-FRET		
Total ratio	Area	Average ratio
4971.4243	6603	0.7529
2172.1596	4227	0.5139
3696.3002	4914	0.7522
4231.499	5399	0.7838
2242.6821	3896	0.5756
6285.4898	6705	0.9374
6401.86	6636	0.9647
3420.9196	4831	0.7081
5571.0691	6210	0.8971
5835.8561	10547	0.5533
5558.5338	6183	0.899
4488.6888	6830	0.6572
4781.5703	7027	0.6805

P-gp-FRET + 100 µM verapamil		
Total ratio	Area	Average ratio
3728.0774	3559	1.0475
2504.558	2563	0.9772
3700.1799	3061	1.2088
3502.4404	4180	0.8379
8828.1776	6599	1.3378
8978.9145	7507	1.1961
8637.2704	10589	0.8157
2187.5205	1582	1.3828
8635.1735	8492	1.0169
6954.1548	6274	1.1084
3580.8059	3299	1.0854
6821.6081	5519	1.236

P-gp-VsCn		
Total ratio	Area	Average ratio
5507.3784	7663	0.7187
13113.399	7994	1.6404
11995.656	9485	1.2647
8018.5248	6770	1.1844
9937.5475	7352	1.3517
6738.6387	7031	0.9584
9195.2399	7271	1.2646
11241.973	7881	1.4265
7788.7173	6600	1.1801
10443.486	7659	1.3636
15673.568	10942	1.4324
11883.661	12093	0.9827
7955.8383	6874	1.1574

P-gp-VsCn + 100 µM verapamil		
Total ratio	Area	Average ratio
11919.1742	7162	1.6642
9605.3738	8383	1.1458
10110.0648	7157	1.4126
6524.1467	7239	0.9012
3267.0807	3355	0.9738
9422.6793	7971	1.1821
2690.3425	3513	0.7658
8544.179	6602	1.2942
10383.7458	7701	1.3484
9469.6927	7739	1.2236
16599.1497	14044	1.1819
11006.3173	12157	0.9053
8937.8437	7201	1.2412

Figure 6. Representative results of the quantification of FRET efficiency. Representative data from cells expressing one type of fluorescent protein-tagged P-gp in the absence or presence of the transport substrate verapamil. Each value was obtained from a single cell. Average ratio indicates the proximity ratio.

```
setBatchMode(true); //Batchmode ON
//setBatchMode(false); //Batchmode OFF

dir1 = getDirectory("Select mVenus folder");
//Select the folder in which the mVenus images are saved
dir2 = getDirectory("Select mCerulean folder");
//Select the folder in which the mCerulean images are saved
dir3 = getDirectory("Select cFRET folder");
//Select the folder in which the cFRET images are saved
dir4 = getDirectory("Select ROI folder");
//Select the folder in which the ROI information is to be saved
dir5 = getDirectory("Select PM folder");
//Select the folder in which the segmented plasma membranes are to be
saved
dir6 = getDirectory("Select ratio image folder");
//Select the folder in which the ratio images are to be saved
```

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```
    list1 = getFileList(dir1);
    list2 = getFileList(dir2);
    list3 = getFileList(dir3);

    //Segmentation of the plasma membrane from a mVenus image

    for (i = 0; i < list1.length; i++){
    open(dir1 + list1[i]);
    Ori_name = getTitle(); //Get name of the image

    run("Duplicate...", Ori_name);
    //For segmentation of the plasma membrane, duplicate the original image
    run("Subtract Background...", "rolling=10");
    //Subtract background. ***Rolling ball radius should be optimized to avoid removing any objects***
    run("Enhance Contrast...", "saturated=0.4 normalize");
    run("Auto Local Threshold", "method=Phansalkar radius=15 parameter_1=0 parameter_2=0 white");
    //***To choose the most suitable method when making a binary image, try all methods (Image > Adjust > Auto Local Threshold > Method "Try all")***
    run("Options...", "iterations=1 count=3 black do=Open");
    //Smoothen the plasma membrane by "Open (Dilute after Erode)"
    run("Analyze Particles...", "size=1-100 pixel include add");
    setForegroundColor(0,0,0);
    roiManager("deselect");
    roiManager("Fill");
    // Delete objects less than 100 pixels
    setOption("BlackBackground", true);
    run("Erode");
    roiManager("Delete");
    run("Analyze Particles...", "size=50-Infinity pixel circularity=0.00-0.3 add");
    // Select objects with size more than 50 pixels and circularity less than 0.3
    run("Select All");
    //Select all ROI
    roiManager("Combine");
    //Combine all ROI
    setBackgroundColor(0, 0, 0);
```


Please cite this article as: Futamata *et. al.*, (2021). Live Cell FRET Analysis of the Conformational Changes of Human P-glycoprotein,*Bio-protocol* 11 (4): e3930. DOI: 10.21769/BioProtoc.3930.


```
run("Clear Outside");
//Delete outside of the ROI in mVenus image
Save_name = dir5 + "mem_" + Ori_name;
saveAs("Tiff", Save_name);
close();

selectImage(1);
setOption("Show All",true);
roiManager("Measure");
Save_name = dir4 + "ROI_" + Ori_name + ".zip";
roiManager("Save", Save_name); //Save ROI information
close();
roiManager("Delete");
}

list4 = getFileList(dir4);

//Evaluation of the proximity ratio from mCerulean and cFRET images

for (i = 0; i < list2.length; i++){
open(dir2 + list2[i]);
//Open mCerulean image
Ori_name = getTitle();
open(dir3 + list3[i]);
//Open cFRET image
run("Images to Stack", "name=Stack title=[] use keep");
setSlice(2);
run("Add Slice");
setSlice(1); //Select mCerulean image
roiManager("Open", dir4 + list4[i]);
run("Select All");
roiManager("Combine");
setBackgroundColor(0, 0, 0);
run("Clear Outside","Slice");
//Delete outside of the ROI in the mCerulean image
for(ii = 0; ii < getWidth(); ii ++){
for(iii = 0; iii < getHeight(); iii ++){
val = getPixel(ii,iii);
if(val > 0){
setSlice(2);
```

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```
    val2 = getPixel(ii,iii);
    ratio = val2 / val;
    //Calculate the proximity ratio (pixel value of cFRET image / pixel
value of mCerulean image)
    sum += ratio;
    count += 1;
    setSlice(3);
    ratiop = ratio * 100;
    setPixel(ii, iii, ratiop);
    setSlice(1);
    }
    }
    }
    updateDisplay();
    Save_name = dir6 + "ratio_" + Ori_name;
    saveAs("Tiff", Save_name);
    ratiom = sum / count;
    //Divide total proximity ratio by the counted pixel number
    ar = newArray(sum, count, ratiom);
    Array.print(ar);
    //show sum of the proximity ratio, pixel numbers of the plasma membrane,
and average of the proximity ratio on the Log window

    sum = 0;
    count = 0;
    run("Close All");
    roiManager("Delete");
    }
```

Recipes

1. PLL solution

0.01% PLL solution	1 ml
1× PBS(-)	29 ml

   Store at 4 °C
2. 10× PBS(-)

   Note: (-) means without magnesium or calcium.

NaCl	80 g
$Na_2HPO_4 \cdot 12H_2O$	29 g
KCl	2 g

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KH_2PO_4 2 g

Fill up to 1 L with milliQ. Store at room temperature

3. 1× PBS(-)

   10× PBS(-) 100 ml

   MilliQ 900 ml

   Sterilize by autoclave

   Store at 4 °C

4. 10× transport buffer

   1 M Hepes-KOH (pH 7.4) 125 ml

   CH_3COOK 56.44 g

   $MgCl_2·6H_2O$ 2.54 g

   Fill up to 500 ml with MilliQ and sterilize with 0.22 μm filter

   Store at room temperature.

5. 1× transport buffer

   10× transport buffer 100 ml

   MilliQ 900 ml

   Store at 4 °C

6. 0.05% Trypsin-EDTA

   0.5% Trypsin-EDTA (10×) 10 ml

   1× PBS(-) 90ml

   Store at 4 °C

7. 100 mM ATP stock solution

   ATP disodium salt 551.1 mg

   Fill up to 10 ml with MilliQ

   Adjust pH to 7 with NaOH

   Sterilize with 0.22 μm filter

   Store at -30 °C

8. 50 mM verapamil stock solution

   Note: Prepare before use.

   Verapamil chloride 24.6 mg

   DMSO 1 ml

   Sterilize with 0.22 μm filter

   Store at -30 °C

9. 1 mg/ml DAPI stock solution

   DAPI 1 mg

   DMSO 1 ml

   Sterilize with 0.22 μm filter

   Store at -30 °C

10. 100 mM NaN_3 stock solution

NaN_3	6.501 mg
MilliQ	1 ml

Sterilize with 0.22 µm filter

Store at -30 °C

11. 1 M $MgCl_2$ stock solution

$MgCl_2 \cdot 6H_2O$	20.33 g

Fill up to 100 ml with MilliQ

Sterilize by autoclave

Store at 4 °C

12. 1 mg/ml PEI-MAX

PEI-MAX	10 mg
1× PBS(-)	10 ml

Sterilize with 0.22 µm filter

Store at 4 °C

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers 18H05269.

Competing interests

The authors declare no competing financial interests.

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