Video Article

Cell-based Calcium Assay for Medium to High Throughput Screening of TRP Channel Functions using FlexStation 3

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Abstract

The Molecular Devices' FlexStation 3 is a benchtop multi-mode microplate reader capable of automated fluorescence measurement in multi-well plates. It is ideal for medium- to high-throughput screens in academic settings. It has an integrated fluid transfer module equipped with a multi-channel pipetter and the machine reads one column at a time to monitor fluorescence changes of a variety of fluorescent reagents. For example, FlexStation 3 has been used to study the function of Ca^{2+}-permeable ion channels and G-protein coupled receptors by measuring the changes of intracellular free Ca^{2+} levels. Transient receptor potential (TRP) channels are a large family of nonselective cation channels that play important roles in many physiological and pathophysiological functions. Most of the TRP channels are calcium permeable and induce calcium influx upon activation. In this video, we demonstrate the application of FlexStation 3 to study the pharmacological profile of the TRPA1 channel, a molecular sensor for numerous noxious stimuli. HEK293 cells transiently or stably expressing human TRPA1 channels, grown in 96-well plates, are loaded with a Ca^{2+}-sensitive fluorescent dye, Fluo-4, and real-time fluorescence changes in these cells are measured before and during the application of a TRPA1 agonist using the FLEX mode of the FlexStation 3. The effect of a putative TRPA1 antagonist was also examined. Data are transferred from the SoftMax Pro software to construct concentration-response relationships of TRPA1 activators and inhibitors.

Video Link

The video component of this article can be found at https://www.jove.com/video/3149/

Protocol

1. Cell preparations in 96-well plates

For HEK293 cells transiently trasfected with human TRPA1 cDNA

1. HEK293 cells are grown for one to two days in Dulbecco's Minimal Essential Medium (DMEM) containing 4.5 mg/ml glucose (Thermo Scientific, SH3002201), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, 16000-044), 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen, 15140-163). On the day of the transfection, the cell density should reach 70-90%.
2. Coat 96-well plates by pipetting 50 µl poly-L-ornithine (Sigma P3655, 20 µg/ml in sterile distilled water) to each well and incubating at 37°C for at least 15 min. Rinse each well once with Dulbecco's Phosphate Buffered Saline (DPBS) without Mg^{2+} and Ca^{2+} (Thermo Scientific, SH3002803). The plate may be left in the cell culture hood for a few hours.
3. For every well of a 96-well plate, dilute in a 1.5-ml Eppendorf tube 0.2 µg cDNA in 25 µl OPTI-MEM (Invitrogen, 31985-070). Scale up proportionally according to the number of wells to be transfected by the same cDNA. Also dilute in a separate 1.5-ml Eppendorf tube 0.4 µl Lipofectamine 2000 (Invitrogen, 11668-019) in 25 µl OPTI-MEM for each well to be transfected. Scale this up according to the total number of wells to be transfected.
4. Briefly vortex the diluted cDNA and Lipofectamine 2000 and let them sit at the room temperature for about 5 min.
5. Transfer an equal volume of the diluted Lipofectamine 2000 to the tube that contains the diluted cDNA, vortex and let the mixture sit at room temperature for 15 min to 2 hrs. During this time, transfer the cDNA/Lipofectamine 2000 mixture at 49 µl/well to the poly-L-ornithine coated plate.
6. Trypsinize the HEK293 cells (Step 1.1), count cell density using a hemocytometer or Countess automated cell counter (Invitrogen, C10227), and transfer a desired amount of cells (generally about ~125,000-150,000 cells/well) to a 15-ml sterile conical-bottom centrifuge tube. Centrifuge at 1000 x RPM for 5 min. Resuspend cells at ~1,250,00-1,500,000 cells/ml in the DMEM culture medium, supplemented with 10% heat-inactivated fetal bovine serum, but without antibiotics.
7. Dispense 98 µl cell suspension to each well that contains the cDNA-Lipofectamine 2000 mixture using a multichannel pipetter or the MicroFill Microplate Dispenser (Bio-Tek). Let the plate sit in the hood for at least 30 min before placing it to the humidified cell culture incubator. Incubate at 37°C, 5% CO_2 for 20-44 hrs.

For HEK293 cells stably expressing TRPA1

8. Follow Step 1.2 to coat 96-well plates.
9. The inducible HEK293-hTRPA1 cell line was generously provided by Dr. A. Patapoutian (The Scripps Research Institute) and maintained in the same medium as the wild-type HEK293 cells (Step 1.1) but supplemented with 5 µg/ml blasticidin (Invitrogen, A1113902) and 50 µg/ml hygromycin B (Invitrogen, 10687010). When cell density reaches about 90%, trypsinize cells, count cell density, and centrifuge the desired amount of cells at 1000 x RPM for 5 min. Resuspend cells at a density of ~1,000,000 cells/ml in the same culture medium supplemented with doxycycline (Fisher, BP26535, 0.25 µg/ml).
10. Dispense the cell suspension to poly-L-ornithine coated plates at 100 µl/well using a multichannel pipetter or the MicroFill (Bio-Tek). Let the plate sit in the hood for at least 30 min before placing it to the humidified cell culture incubator. Incubate at 37 °C, 5% CO_2 for 16 - 24 hr.

2. Solutions

1. Hank's buffered salt solution (HBSS) is used as the extracellular solution. It contains (in mM): 137 NaCl, 5.4 KCl, 0.25 Na_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 1.0 $MgSO_4$, 1.0 $MgCl_2$, 10 glucose, 10 HEPES, with pH adjusted to 7.4 using 1N NaOH).
2. Fluo-4 assay buffer contains 2 mM probenecid in HBSS. Make 250 mM stock probenecid (Sigma, P8761) in 0.5 N NaOH. Dilute to HBSS at a ratio of 1:125. Readjust pH to 7.4 using 1 N HCl.

3. Cell loading with Fluo-4 AM

1. Dissolve 1 mg Fluo-4 AM (Invitrogen, F14202) in 912 µl anhydrous DMSO to make a stock solution of 1 mM. The Fluo-4 AM stock solution can be stored at -20 °C for at least one month.
2. Mix 10 µl 1 mM Fluo-4 AM with 10 µl Pluronic F-127 (Invitrogen, P3000MP, 20% (w/v) solution in DMSO) and then transfer the entire content to 5 ml Fluo-4 assay buffer (Step 2.2) supplemented with 0.1% Bovine serum albumin (Sigma, A9418).
3. Wash cells grown in 96-well plates (Step 1.7 or 1.10) with HBSS at 80 µl/well 2 times using a microplate washer (such as ELx405TM from Bio-Tek).
4. Add the Fluo-4 AM loading solution (Step 3.2) at 50 µl/well using a multichannel pipetter or the MicroFill (Bio-Tek) and incubate the plate at 37 °C for 1 hr.
5. Wash cells 3 times with the Fluo-4 assay buffer (Step 2.2.) using the ELx405TM microplate washer. After the last wash, leave 80 µl of the Fluo-4 assay buffer in each well.

4. Preparation of compound plate

1. During the 1 hr cell loading period, prepare a series of 3x dilutions of agonists (or antagonists) of 3x of the desired final concentrations in the Fluo-4 assay buffer.
2. Pipette 250-300 µl of the diluted compound solutions to a 96-well compound plate. Arrange to have the serial dilutions of a compound and the corresponding positive and negative controls in the same column of the compound plate whenever it is possible because FlexStation 3 reads one single column at a time.

5. Plate reading in FlexStation 3

1. Turn on the system (FlexStation 3, computer, and the SoftMax Pro 5.2 software)
2. Save the experiment with a new file name. At the set-up, choose FLEX mode and enter the following values:

Protocol #1, agonist effect

a.	Wavelengths	
	Excitation	494
	Emission	525
	Emission cutoff	515
b.	Sensitivity	
	Readings	3
	PMT	medium
c.	Timing	
	Time	180 s
	Interval	~1.5 s (but can also try 2-5 s)
d.	Assay plate type	Greiner 96-well black/U clear bottom
e.	Wells to read	Columns 1-12 (or other values)
f.	Compound source	Costar 96 well clear flat bottom
g.	Compound transfer	
	Transfers	1
	Initial volume	80 µl
	Pipettor height	110 µl
	Transfer volume	40 µl
	Dispense speed	3
	Transfer times	30 s
h.	Pipette Tips Layout	depending on experimental design
i.	Compound & Tip Column	depending on experimental design

Protocol #2, antagonist effect

a.	Wavelengths	
	Excitation	494
	Emission	525
	Emission cutoff	515
b.	Sensitivity	
	Readings	3
	PMT	medium
c.	Timing	
	Time	300 s
	Interval	~1.5 s (but can also try 2-5 s)
d.	Assay plate type	Greiner 96-well black/U clear bottom
e.	Wells to read	Columns 1-12 (or other values)
f.	Compound source	Costar 96 well clear flat bottom
g.	Compound transfer	
	Transfers	2
	Initial volume	80 µl
	Pipettor height	110 µl for first, 170 µl for second transfer
	Transfer volume	40 µl for first, 60 µl for second transfer if needed
	Dispense speed	3
	Transfer times	30 s for the first transfer, 180 s for the second transfer
h.	Pipette Tips Layout	depending on experimental design
i.	Compound & Tip Column	depending on experimental design

3. Place a new tip box, compound plate and the reading plate to the appropriate trays in FlexStation 3 and click "read" button. The FlexStation 3 will read one column at a time and add compounds at the preprogrammed time points without interrupting the reading. It takes about 3 min (5 min for Protocol #2) to finish reading one column and the machine will continue to read the next column with compound additions performed as programmed by the user in step 5.2 until all columns are read.

6. Data analysis

1. Experimental data may be processed directly using the SoftMax Pro 5.2 software or copied and pasted into any spreadsheet program, such as Microsoft Excel. Concentration-response curve of a TRPA1 agonist flufenamic acid (FFA) is constructed using the following least-squares fitting routine. $Y = Y_{max}/[1 + (C/EC_{50})^{n_H}]$, where Y is the observed response, Y_{max} is the normalized maximal response, C is the corresponding drug concentration, EC_{50} is the concentration that evoked a half-maximal response, and n_H is the apparent Hill coefficient. Concentration-inhibition curve for a putative TRPA1 antagonist compound A is obtained by using a fixed concentration of FFA (100 µM), and progressively increasing the concentration of compound A. IC_{50} value of compound A is calculated by least-squares fitting to the following equation. $Y = Y_{max}/[1 + (IC_{50}/[Ant])^{n_H}]$, where Y is the observed response and Y_{max} is normalized peak response, [Ant] is the corresponding compound A's concentration, IC_{50} is the antagonist concentration that produces half-maximal inhibition of FFA-evoked response, and n_H is the apparent Hill coefficient.

7. Representative Results:

TRPA1 is a Ca^{2+}-permeable cation channel, activation of which leads to calcium influx that can be detected by the Ca^{2+}-sensitive dye, Fluo-4 (1-2). A HEK293-hTRPA1 stable cell line was used to study the concentration-response relationships of TRPA1 agonists and antagonists. Cells were loaded with Fluo-4 AM, placed in 80 µl of the assay buffer, and read using FlexStation 3. After recording the baseline fluorescence for 30 s, 40 µl of a serial dilution of the TRPA1 agonist, FFA, each at 3x of the desired final concentration, were added to the cells through a multi-channel pipetter included as a part of the fluidics module of FlexStation 3. The fluorescence changes were monitored for an additional 180 s. The relative Fluo-4 fluorescence changes for each well were expressed as $\Delta F = (F - F_0)$, where F and F_0 are fluorescence values at the time of interest and the beginning of the reading, respectively. Representative traces of the time courses to fluorescence changes in response to a series of FFA concentrations are shown in Fig. 1. Note for the last four FFA concentrations, although the maximal response levels are similar, the activation kinetics is clearly faster at the higher than at the lower FFA concentrations. Therefore, to distinguish these differences, the concentration - response relationship was constructed by measuring the initial rates (slope) of the fluorescence increase upon addition of varying concentrations of FFA (Fig. 2). The half maximal effective concentration (EC_{50}) of FFA was determined to be 55.4 µM (n=3). To test the putative TRPA1 antagonist, compound A, we performed a similar experiment using protocol #2 (Step 2). In this case, a series of dilutions of the antagonist were added at 30 s (40 µl of 3x desired final concentrations) and the FFA was added 180 s later (60 µl at 300 µM for a final

concentration of 100 µM). Compound A dose-dependently reduced the response of the TRPA1 cells to FFA with a half maximal inhibitory concentration (IC_{50}) of 4.3 µM (n=3) (Fig. 2).

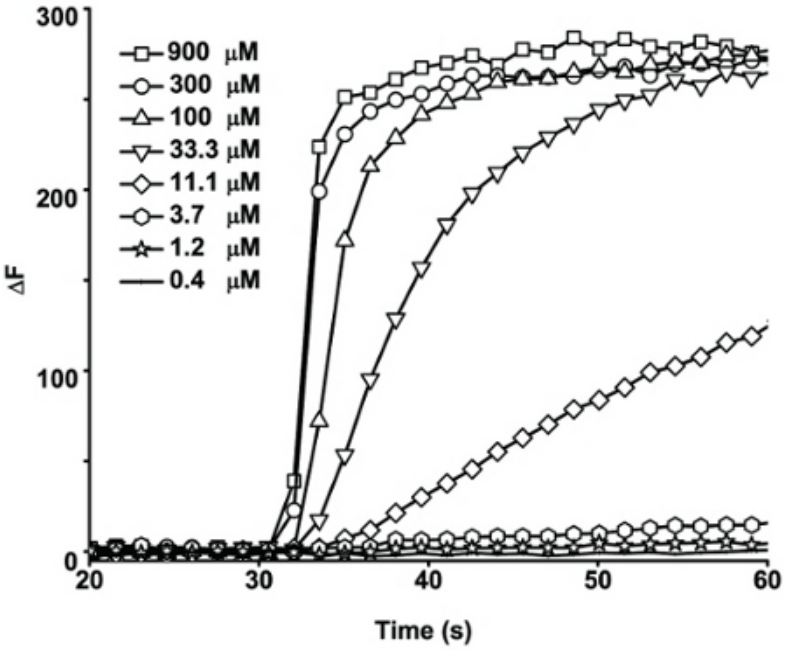

Figure 1. Concentration-dependent activation of human TRPA1 by FFA. Traces are FFA-evoked increase of fluorescence over time (only responses in the first 60 s are shown to better illustrate the fast kinetics of the FFA-evoked responses). Please note that at higher concentrations FFA evoked fluorescence increase with a much faster kinetic than at lower concentrations, even though the peak fluorescence levels were similar.

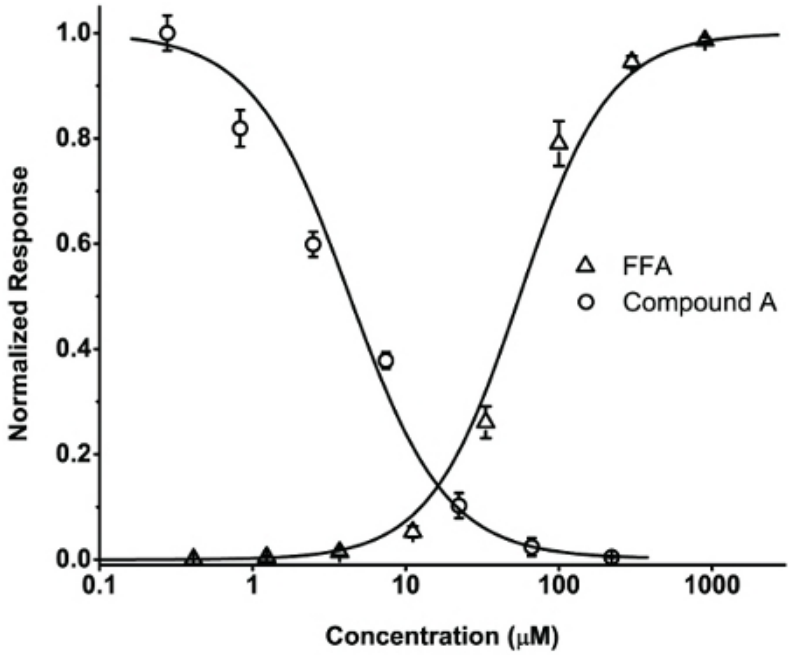

Figure 2. Concentration - response relationships for FFA-induced fluorescence change and compound A-induced inhibition of FFA response in human TRPA1 stably expressed in HEK293 cells. Δ,The concentration - response relationship of FFA was determined by measuring the initial rates (slope) of the normalized fluorescence increase upon addition of varying concentrations of FFA. O, Concentration-response relationship of inhibition of FFA-evoked calcium response by a putative TRPA1 antagonist, compound A.

Discussion

Intracellular Ca^{2+} plays important roles in many physiological and pathological conditions, such as neurotransmitter release (3), cardiac action potential (4), muscle cell contraction (5), cell signal transduction (6-7), and excitotoxic cell death (8). Measurement of intracellular Ca^{2+} levels helps to elucidate functional changes of Ca^{2+}-permeable channels in the plasma membrane or intracellular organelles and the contribution of

Ca^{2+} to the above processes (9-13). The assay can also be used to investigate the function of G-protein coupled receptors, many of which, upon activation, lead to increased intracellular free Ca^{2+} concentrations by releasing Ca^{2+} from intracellular Ca^{2+} stores or activating other Ca^{2+} permeable ion channels (14-16). FlexStation 3 makes use of Ca^{2+} sensitive dyes, which give rise to increased fluorescence intensity, or ratiometric changes, with the increase of intracellular Ca^{2+} concentration. This video article demonstrates the application of FlexStation 3 in the study of Ca^{2+}-permeable TRPA1 channels heterologously expressed in HEK293 cells. Although we have only used 96-well plates for the demonstration, users can easily choose from 96- or 384-well plate format by switching the dispensing pipetters (8- or 16-channel). This assay provides a quick medium to high throughput screening capability of agonists and/or antagonists and is applicable to many other Ca^{2+}-permeable ion channels. However, it should be noted that intracellular calcium assay is an indirect measurement of ion channel activities. Detailed follow-up studies using patch clamp recordings to directly measure ionic current flowing across cell membrane are still needed to draw appropriate conclusions.

To obtain reliable data quality, the following precautions should be taken:

1. For loosely adherent cells, such as HEK293 cells, coating the plates is essential. However, for strongly adherent cells, such as Chinese Hamster Ovary (CHO) cells or HELA cells, coating is not necessary.
2. It is important that equal amount of cells are seeded in each well of the multi-well plate and the cell density in each well should be between 90-100% confluency at the time of the assay.
3. Gently dispense liquid to the cells and avoid disturbing the cells when loading and washing cells.
4. Probenecid is added to prevent dye leakage. However, since probenecid also has effect on some ion channels, caution should be taken when interpreting data obtained from experiments that include probenecid in the assay buffer.
5. To determine the compound concentrations in the compound plate, consider the dilution factor for each well according to the buffer volume in the corresponding well of the sample plate before and after the compound addition.

Disclosures

No conflicts of interest declared.

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