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Fluorescence-based measurements of store-operated Ca^{2+} entry in cancer cells using Fluo-4 and confocal live cell imaging

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Abstract

The store-operated calcium entry (SOCE) is the predominant calcium entry mechanism in cancer cell and other non-exciting cells. In the last few years, there is rapidly accumulating evidence supporting that SOCE is dysregulated in many cancers. The hyperactive SOCE in tumor cells is spatially and temporally coded to promote cell proliferation, migration and invasion. In this chapter, we describe two protocols to measure SOCE in tumor cells. The first protocol employs fluorescent microplate readers and could be adapted for high throughput screening. The second protocol takes advantage of laser scanning confocal microscopy and can be used to resolve the high resolution spatial and temporal coding of SOCE signals in single cells. These protocols are useful tools to uncover the dysregulation of SOCE signaling in tumor malignancy.

Keywords

STIM1; Orai1; Store-operated calcium entry; calcium oscillation; cancer cell migration; metastasis; invasion

1. Introduction

Store-operated calcium entry (SOCE) is a Ca^{2+} entry mechanism regulated by extracellular stimuli (1). SOCE is the major Ca^{2+} entry mechanism in non-excitable cells, including most cancer cells (2, 3). It is reported that Stim1 and Orai1, molecular components of store-operated calcium channels, are required for cancer cell migration and breast cancer metastasis through modulating focal adhesion turnover (4). The dysregulation of Stim and/or Orai proteins mediated Ca^{2+} signaling have also been implicated in the migration, invasion and progression of various other cancers (5–16). More recently, temporal organization of

store-operated calcium signal into oscillatory signal is found to be crucial for melanoma invasion and the tumor growth of head and neck cancer (17, 18).

In our lab we use two fluorescence-based methods to measure store-operated calcium entry in cancer cells using fluorescent microplate reader and confocal microscopy (4, 18–20). The microplate method can be conveniently set up using plate reader equipped with kinetic reading function. Since the readout reflects the average Ca^{2+} signal of all the cells in the well, the data processing is straightforward. Furthermore, since many wells can be monitored at the same time, this method can be adapted for high throughput screening assay. The second method involve single cell imaging using confocal microscope equipped with live cell imaging chamber and perfusion device. Confocal microscopy provides excellent spatial and temporal resolution to interrogate the intricate subcellular organization of Ca^{2+} signals (Figure 1).

2. Materials:

1. Fluo4-AM solution: prepare a 2 to 5 mM stock solution with 20% Pluronic F-127 (FluoProbes) dissolved in high-quality, anhydrous DMSO.
2. Tyrode Solution (T.S.): 137 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 10 mM glucose, and 20 mM Hepes (pH 7.35, adjusted with NaOH).
3. Probenecid, 0.2 M stock solution, dissolved in 1 N NaOH (*see Note 1*)
4. Standard Solution (S.S.): 20 mM HEPES (pH 7.4), 130 mM NaCl, 2 mM CaCl_2 , 5 mM KCl, 10 mM glucose, 0.45 mM KH_2PO_4 , 0.4 mM Na_2HPO_4 , 1.2 mM MgSO_4 , 1.2 mM MgCl_2 , 4.2 mM NaHCO_3 , 2.5 mM probenecid, and 0.1% bovine serum albumin.
5. EGTA, 0.5 M stock solution
6. Thapsigargin, 2 mM stock solution in DMSO.

3. Methods:

3.1 Measurement of store-operated calcium influx using microplate reader

1. First day: Cells are plated onto 96-Well clear bottom black plates at a pre-determined optimal density (*see Note 2*).
2. Second day: Cells are washed with S.S. once and incubated with 100 μl /well of 4 μM Fluo-4-AM (diluted in S.S.) for 45 min at room temperature. Use aluminum foil cover to keep the plate in the dark (*see Note 3*).
3. Transfer the plate to a 37°C incubator and incubate for 15 min (*see Note 4*).

¹Probenecid is not soluble in dH_2O or buffer with neutral pH.

²The optimal number of cells to be plated on to each well is cell type dependent and need to be determined empirically. The key is for the cells to reach about 90% confluence at the time of calcium assay. As example, for WM793 melanoma cells, 15000 cells are plated per well.

³The AM ester is removed by esterase in the cytosol, which will prevent the loading of the dye to intracellular organelle. It is believed that Fluo-4-AM loading at room temperature helps to prevent intracellular organelle loading.

⁴Incubation at 37°C for 15 min facilitates removal of AM ester.

4. Wash the cells with ice cold calcium free S.S. (200 μ L/well) for three times on ice (*see* Note 5).
5. Add 100 μ L calcium free S.S. to each well and record the basal Fluo-4 fluorescence using a fluorescent microplate reader for 30 seconds (1 read per second).
6. Add 2 μ M thapsigargin to induce Ca^{2+} release from the ER. Continue to monitor the change in fluorescence for 300 seconds or until the Fluo-4 fluorescence reach the basal level.
7. Add Ca^{2+} to a final concentration of 2 mM and immediately monitor the change in fluorescence continuously for 300 second.

3.2 Single cell Ca^{2+} measurement using confocal microscopy

3.2.1 Single cell measurement of Ca^{2+} oscillation

1. Plate cells on glass-bottom 35mm petri dishes or POC system compatible glass coverslips 24–48h before imaging.
2. Discard culture medium and wash cells twice with 1 mL pre-heated (37°C) T.S. containing 2 mM Ca^{2+} .
3. Load cells with 400 μ L T.S. containing 2 mM Ca^{2+} and 5 μ M Fluo4-AM for 10 min at room temperature or 37°C.
4. Wash cells with T.S. containing 0 Ca^{2+} (5 mM EGTA included) or 2 mM Ca^{2+} twice after Fluo-4-AM loading (*see* Note 6).
5. Add 400 μ L or more new T.S. containing 0 Ca^{2+} (5 mM EGTA included) or 2 mM Ca^{2+} for imaging.
6. Confocal imaging is carried out with an inverted Zeiss LSM710 microscope with a 40 $\times$, 1.3 N.A. oil immersion objective. Cells are kept in a Heating Insert P S1 incubator (Pecon) at a constant temperature of 37°C under 5% CO_2 (vol/vol).
7. Apply 488 nm wavelength to excite the samples and collect fluorescence emission at > 505 nm for single color calcium imaging.
8. Set the pinhole for a nominal 1.2 μ m optical section. Adjust gain and laser intensity to obtain good images. Note that these imaging parameters can be adjusted according to the signal noise ratio and experimental conditions. Keep identical for all samples once determined.
9. Use definite focus to ensure the stability of the focal plane over time.
10. Apply the sampling speed of 0.5 ~ 2 s/frame (512 $\times$ 512 pixels) with 5 ~15 s intervals for 1 h to capture the calcium dynamics. Scan the samples first for 1~3

⁵ Some cells might be sensitive to washing with calcium free S.S. If cell detachment becomes an issue, regular S.S. instead of S.S. can be used. After the final wash, EGTA can be added to chelate the Ca^{2+} ion in buffer.

⁶ 0 Ca^{2+} (5 mM EGTA included) is used to eliminate the calcium influx mediated by CRAC channel. The strategy of 0 Ca^{2+} switching to 2 mM Ca^{2+} or 2 mM Ca^{2+} switching to 0 Ca^{2+} can be adopted according to experimental requirements.

min baseline. 10% FBS (or other treatment) are added through MPS-2 multichannel perfusion system connected to the infusion chamber to stimulate Ca^{2+} oscillation. The Ca^{2+} oscillation in these cells are continuously monitored for typically 1 hour at the same sampling speed.

11. When necessary, pharmacological inhibitors for CRAC channels, EGTA and Ca^{2+} can be added to the cell at various stage of the experiment through the perfusion system to study the role of extracellular Ca^{2+} and CRAC channels in Ca^{2+} oscillation.

3.2.2 Data analysis—Digital images are processed using customer-devised routines written in IDL (ITT, New York, NY) (*see* Note 7).

1. Circle out the cells along their outlines and output the traces of time series in a txt format.
2. Run the IDL program and it returns each trace figure and peak number of all the cells selected.
3. Check each trace combined with original images of time series. Recover the missing peaks and remove the error due to the ups and downs of baseline.
4. Collect the peak number per hour (e.g. frequency of calcium oscillations) and do comparison between different groups.

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References

1. Putney JW, Jr. (1986) A model for receptor-regulated calcium entry. *Cell calcium*. 7(1), p. 1–12. [PubMed: 2420465]
2. Hogan PG, Lewis RS, Rao A (2010), Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annual review of immunology* 28, p. 491–533.
3. Prevarskaya N, Skryma R, Shuba Y (2011) Calcium in tumour metastasis: new roles for known actors. *Nature reviews. Cancer* 11(8), p. 609–18. [PubMed: 21779011]
4. Yang S, Zhang JJ, Huang XY (2009) Orail and STIM1 are critical for breast tumor cell migration and metastasis. *Cancer Cell* 15(2), p. 124–34. [PubMed: 19185847]
5. Wang JY, Chen BK, Wang YS, et al. (2012) Involvement of store-operated calcium signaling in EGF-mediated COX-2 gene activation in cancer cells. *Cellular signalling* 24(1), p. 162–9. [PubMed: 21924350]
6. Hou MF, Kuo HC, Li JH, et al. (2011), Orail/CRACM1 overexpression suppresses cell proliferation via attenuation of the store-operated calcium influx-mediated signalling pathway in A549 lung cancer cells. *Biochimica et biophysica acta* 1810 (12), p. 1278–84. [PubMed: 21782006]
7. Berry PA, Birnie R, Droop AP, et al. (2011) The calcium sensor STIM1 is regulated by androgens in prostate stromal cells. *The Prostate* 71(15), p. 1646–55. [PubMed: 21432868]

⁷ Principles of peak finding (IDL program): The program finds local maxima or peaks in fluorescent intensity F, which must contain seven sampling points. Each value of F is compared to its neighboring values, and if it is larger than both of its neighbors, it is a local peak and is returned. If no peaks are found, return 0.

8. Chen YF, Chiu WT, Chen YT, et al. (2011) Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. *Proceedings of the National Academy of Sciences of the U S A*. 108(37), p. 15225–30.
9. Fedida-Metula S, Feldman B, Koshelev V, et al. (2012) Lipid rafts couple store-operated Ca^{2+} entry to constitutive activation of PKB/Akt in a Ca^{2+} /calmodulin-, Src- and PP2A-mediated pathway and promote melanoma tumor growth. *Carcinogenesis* 33(4), 740–50. [PubMed: 22287561]
10. Hu J, Qin K, Zhang Y, et al. (2011) Downregulation of transcription factor Oct4 induces an epithelial-to-mesenchymal transition via enhancement of Ca^{2+} influx in breast cancer cells. *Biochemical and biophysical research communications* 411(4), p. 786–91. [PubMed: 21798248]
11. Huang WC, Chai CY, Chen WC, et al. (2011) Histamine regulates cyclooxygenase 2 gene activation through Orai1-mediated NF κ B activation in lung cancer cells. *Cell calcium* 50(1), p. 27–35. [PubMed: 21605904]
12. Yang N, Tang Y, Wang F, et al. (2012) Blockade of store-operated Ca^{2+} entry inhibits hepatocarcinoma cell migration and invasion by regulating focal adhesion turnover. *Cancer letters* 330(2), 163–9. [PubMed: 23211538]
13. Ryu S, McDonnell K, Choi H, et al. (2013) Suppression of miRNA-708 by Polycomb Group Promotes Metastases by Calcium-Induced Cell Migration. *Cancer Cell* 23(1), p. 63–76. [PubMed: 23328481]
14. McAndrew D, Grice DM, Peters AA, et al. (2011) ORAI1-mediated calcium influx in lactation and in breast cancer. *Molecular cancer therapeutics* 10(3), p. 448–60. [PubMed: 21224390]
15. Wang JY, Sun J, Huang MY, et al. (2015) STIM1 Overexpression Promotes Colorectal Cancer Progression, Cell Motility and COX-2 Expression. *Oncogene* 34(33), 4358–67.
16. Stanis H, Saul S, Müller CS, et al. (2014) Inverse regulation of melanoma growth and migration by Orai1/STIM2-dependent calcium entry. *Pigment Cell Melanoma Res* 27(3), p. 442–53. [PubMed: 24472175]
17. Zhu H, Zhang H, Jin F, et al. (2014) Elevated Orai1 expression mediates tumor-promoting intracellular Ca^{2+} oscillations in human esophageal squamous cell carcinoma. *Oncotarget* 5(11), p. 3455–71. [PubMed: 24797725]
18. Sun J, Lu F, He H, et al. (2014) STIM1- and Orai1-mediated Ca^{2+} oscillation orchestrates invadopodium formation and melanoma invasion. *J Cell Biol* 207(4), p. 535–48. [PubMed: 25404747]
19. Yang S, Huang XY (2005) Ca^{2+} Influx through L-type Ca^{2+} Channels Controls the Trailing Tail Contraction in Growth Factor-induced Fibroblast Cell Migration. *J Biol Chem* 280(29): p. 27130–7. [PubMed: 15911622]
20. Wei C, Wang X, Chen M, et al. (2009) Calcium flickers steer cell migration. *Nature* 457(7231), p. 901–5. [PubMed: 19118385]

Figure 1. Ca^{2+} oscillation in WM793 cells revealed by confocal. A, montage of live-cell Ca^{2+} imaging showing oscillatory Ca^{2+} signals in control WM793 cells induced by stimulation with 10% FBS. B, shRNA knockdown of STIM1 and Orai1 in WM793 cells abrogate the Ca^{2+} oscillation after FBS treatment. The intervals between adjacent frames are 2 second.