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# Monitoring VEGF-stimulated calcium ion flux in endothelial cells

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**Running title: Monitoring endothelial calcium flux**

## Abstract

The endothelial response to vascular endothelial growth factor A (VEGF-A) regulates many aspects of animal physiology in health and disease. Such VEGF-A-regulated phenomena include vasculogenesis, angiogenesis, tumor growth and progression. VEGF-A binding to receptor tyrosine kinases such as vascular endothelial growth factor receptor 2 (VEGFR2) activates multiple signal transduction pathways and changes in homeostasis, metabolism, gene expression, cell proliferation, migration and survival. One such VEGF-A-regulated response is a rapid rise in cytosolic calcium ion levels which modulates different biochemical events and impacts on endothelial-specific responses. Here, we present a series of detailed and robust protocols for evaluating ligand-stimulated cytosolic calcium ion flux in endothelial cells. By monitoring an endogenous endothelial transcription factor (NFATc2) which displays calcium-sensitive redistribution, we can assess the relevance of cytosolic calcium to protein function. This protocol can be easily applied to both adherent and non-adherent cultured cells to evaluate calcium ion flux in response to exogenous stimuli such as VEGF-A.

**Key words** Endothelial cells, calcium, VEGF-A, VEGFR2, NFATc2, Human umbilical vein endothelial cells (HUVECs)

## 1 Introduction

The calcium ion is a noted second messenger in many biological systems, especially in eukaryotes. Many cellular proteins with calcium-binding properties have specific domains or motifs that can bind calcium ions reversibly. Such binding can trigger conformational changes resulting in changes in protein activity, distribution and/or interactions with other factors. In this way a wide array of biochemical reactions within many biological systems are modulated by the presence or absence of calcium ions. In many eukaryote cells, calcium ions are stored in intracellular, membrane-bound compartments such as the endoplasmic reticulum (ER). The activation of signal transduction pathways frequently impact on cytosolic calcium ions: this is caused by the activation of inositol-1,4,5-triphosphate receptor (IP3R) which is a membrane protein channel located within the ER [1]. The production of IP3 through signaling events at the plasma membrane, results in rapid binding of IP3 to IP3R to trigger opening of the membrane channel and rapid movement of calcium ions from the ER to the cytosol [2]. In this way, signal transduction pathways frequently trigger cytosolic calcium ions which then impact on different aspects of cellular physiology and pathophysiology [3].

The phenomenon of angiogenesis is the sprouting of new blood vessels from pre-existing ones [4]. The endothelium is a cell monolayer which lines all blood vessels and is a critical interface between circulating blood and the blood vessel wall. The presence of soluble pro-angiogenic factors in circulating fluids e.g. blood, causes endothelial cells to carry out this unique process of angiogenesis. One such family of pro-angiogenic molecules are the VEGF family comprised of A, B, C, D and placental growth factor, PlGF [5,6]. VEGF-A is the most intensively studied and is essential for both vasculogenesis and angiogenesis [7]. Deregulation of angiogenesis linked to increased levels of VEGF-A is involved in different pathological states such as diabetic retinopathy and tumor development [4,7].

VEGF-A binds to the receptor tyrosine kinase VEGFR2 present on endothelial cells to trigger different signaling events [6]. Notably, the canonical mitogen-activated protein kinase (MAPK), p38 MAPK, AKT and JNK pathways are stimulated by the activation of VEGFR2 [6]. One well-established

consequence of VEGF-A binding to human VEGFR2 is autophosphorylation of residue Y1175: this phospho-epitope within the VEGFR2 cytoplasmic tail forms a binding site for a phospholipase, PLCγ1 [8], which is essential for regulating vascular development [9]. PLCγ1 contains Src-homology 2 (SH2) domains which specifically recognize the VEGFR2-pY1175 epitope enabling interaction with activated VEGFR2 [10]. The recruitment of phospholipases such as PLCγ1 triggers hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) located on the cytosolic leaflet of the plasma membrane bilayer. PIP2 breakdown to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) generates two different types of second messengers which can now modulate a wide range of cellular events. IP3 diffuses rapidly throughout the cell and a major target is the ER-bound IP3R ion channel, which results in rapid calcium ion efflux into the cytosol. DAG binds to protein kinase C (PKC) enzymes, triggering PKC activation and phosphorylation of a wide range of cellular targets, including protein kinases within different signal transduction pathways.

The binding of VEGF-A to endothelial VEGFR2 promotes a rapid rise in cytosolic calcium ions [11]. In many immune and hematopoietic-derived tissues including the endothelium, a rise in cytosolic calcium ions leads to activation of a family of transcription factors termed nuclear factor of activated T-cells (NFAT) [12]. The rise in calcium activates a calcium-binding protein called calcineurin, which is a calcium-regulated protein phosphatase. Inactive NFAT is usually phosphorylated on multiple serine and threonine residues and trapped in the cytosol. Upon activation of calcineurin by calcium ions, dephosphorylation of NFAT can occur. Dephosphorylated NFAT is now competent to translocate through nuclear pores into the nucleus. Here, NFAT can now promote gene transcription at multiple gene loci. In endothelial cells, such a pathway has been well-established: VEGF-A binding leads to dephosphorylation of NFATc2 (NFAT1) and translocation into the nucleus [13-15]. The functional target of endothelial NFATc2-regulated gene transcription is unclear, but affects cell migration and not proliferation [15]. Such findings suggest that more detailed studies are needed to provide a mechanism for how VEGF-A regulates calcium-regulated cellular responses in endothelial cells. In this chapter, we provide a rapid and effective protocol for monitoring cytosolic calcium ion flux in response to exogenous ligand such as VEGF-A.

## 2 Materials

Buffers and reagents of analytical grade should be used. Working buffers used sterilized autoclaved double distilled water (purified deionized water with a specific resistance of 18 MΩ/cm$^2$ at 25 °C).

### 2.1 Endothelial cell culture

1. Complete endothelial cell growth medium (ECGM; Promocell, Heidelberg, Germany; Cat. No. C-22010): 500 mL endothelial cell basal medium supplemented with 2 % (v/v) fetal calf serum, 0.4% (v/v) endothelial growth supplement, 0.1 ng/mL epidermal growth factor (EGF), 1 ng/mL basic fibroblast growth factor (bFGF), 90 µg/mL heparin and 1 µg/mL hydrocortisone; all pre-warmed to 37°C before mixing under sterile conditions.
2. TrypLE™ Express (1x), no phenol red
3. Human umbilical vein endothelial cells (HUVEC), see Note 1.
4. Improved Neubauer hemocytometer or similar.
5. 0.1 % (w/v) porcine skin gelatin in phosphate buffered saline (PBS), see Note 2.
6. Phosphate-buffered saline: 140 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, pH 7.3. Phosphate-buffered saline tablets can be used by dissolving one tablet in 500 mL double-distilled water and autoclaved on standard wet cycle at 121 °C.
7. Tissue-culture grade sterile T75 plastic flasks.
8. Gelatin-coated 13 mm glass coverslips.
9. Superfrost glass slides.
10. Mounting medium suitable for fluorescence samples e.g. Fluoromount G mounting medium or similar

**2.2 Calcium flux assay**

1. Tissue-culture grade 96-well plates.
2. SBS buffer (prepared fresh): 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 8 mM glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, pH 7.4.
3. 20 % (w/v) pluronic F-127 in DMSO.
4. Fura-2 AM made to 1 mM in DMSO.
5. VEGF-$A_{165a}$ ligand (Promocell, Heidelberg, Germany; Cat. No. C-64423) supplied as a lyophilized powder. Resuspend at a 1000x stock concentration of 25 μg/mL in sterile PBS pH 7.4 and store at -80 °C in 50 μl aliquots.

**2.3. NFATc2 localisation**

1. Tissue-culture grade sterile 24-well plates.
2. Serum starvation medium (MCDB131/BSA): MCDB131 medium, no glutamine, supplemented with 0.2 % (w/v) BSA and 0.22 μm filter sterilized in a sterile environment; pre-warmed to 37 °C.
3. Fixative: 3 % (w/v) paraformaldehyde (PFA) in PBS containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ (see Note 3).
4. Permeabilization buffer: 0.2 % (v/v) Triton X-100 in PBS pH 7.4.
5. Blocking buffer: 0.5 % (w/v) BSA in PBS pH 7.4.
6. Primary antibody: rabbit monoclonal antibody D43B1 anti-NFATc2/NFAT1 (Cell Signalling Technology, Danvers, USA; Cat. No. 5861).

7. Secondary antibody: AlexaFluor594-conjugated donkey anti-rabbit secondary antibody.
8. 50x stock solution (100 mg/mL) of 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) made up in PBS pH 7.4.

### 2.4. Equipment and software

1. Programmable fluorescence microplate reader with automated on-board pipettor capable of real-time fluorescence monitoring. The instrument must be capable of simultaneous dual excitation at both 340 nm and 380 nm whilst recording emission at 510 nm.
2. Inverted digital fluorescence microscope
3. NIH Image J software with Coloc 2 plugin downloaded from https://imagej.nih.gov/ij/download.html.

# 3 Methods

## 3.1 Primary endothelial cell culture

1. Coat T75 tissue culture flasks with 10 mL of 0.2 % porcine skin gelatin solution and disperse so that the entire base of the flask is covered.
2. Incubate at 37 °C for at least 1 h.
3. Aspirate gelatin solution and wash flask 3 times with sterile PBS and allow the flasks to air dry in tissue culture laminar flow hood.
4. Seed HUVECs into gelatin-coated T75 tissue culture flask at 5,000-10,000 cells per $cm^2$ in ECGM (pre-warmed to 37°C).
5. Incubate at 37 °C until approximately 80 % confluent. Perform media changes as required (see Note 4).
6. Once HUVEC flask reaches 80 % cell confluency, detach cells by aspirating ECGM and replacing with 3mL TrypLE™ Express per T75 flask. Tip the flask to each side to ensure the trypsin solution is able to cover all of the flask surface. Once the cells are coated with TrypLE™ solution, immediately aspirate and repeat a second time with a further 3 mL TrypLE™. Aspirate solution again and incubate cells at 37 °C for up to 5 mins, checking for cell detachment every minute. Stop the trypsin activity by adding 1 mL ECGM. Resuspend HUVECs in ECGM and seed new T75 flasks (if passaging) or appropriate tissue culture plates (for experimental use) at 5,000 – 10,000 cells per $cm^2$. Allow to grow for at least 24 h before performing experiments at approximately 80% confluency. See Note 5.

**3.2. Preparation of HUVECs and loading cells with fluorescent dye.**

1. Coat 96-well tissue culture plates with 100 µl gelatin solution to allow coating and drying as previously described in 3.1.1.
2. Detach 80 % confluent HUVECs with TrypLE™ Express as previously described in 3.1.6
3. Count the number of cells per mL using a Neubauer hemocytometer.
4. Seed HUVECs onto gelatin-coated 96-well plates such that they are fully confluent on the planned day of experiment. For example, seeding 25,000 HUVECs per well enables experiments to be carried out in 48 h or 19,000 HUVECs per well enables experiments to be carried out in 72 h.
5. Incubate cells at 37 °C until fully confluent.
6. Aspirate media and wash cells twice with 100 µl SBS.
7. Prepare dye loading solution in SBS from stock solutions. Dilute stock solutions of Pluronic acid F-127 1:2000, and Fura-2 AM 1:500 to obtain a final working solution containing 0.01 % Pluronic F-127 and 2 µM Fura-2 AM.
8. Load HUVECs with 50 µl per well of Fura-2 AM/Pluronic F-127/SBS (2µM Fura-2 AM, 0.01 % Pluronic F-127) by incubation for 60 min at 37 °C.

**3.3 Cytosolic calcium flux assay.**

1. Wash HUVECs with 100 µl SBS. For addition of a single compound or multiple compounds at a single time point, wash a second time with 200 µl of SBS. Incubate cells for 30 min at room temperature to allow complete de-esterification of Fura-2 AM.
2. Prepare compound and/or ligand(s) to be added (e.g. VEGF-A) diluted to a 5x stock concentration in a 96-well U-bottom plate. Addition of solutions to the HUVEC cells will result

in a 1:5 dilution. Prepare a volume sufficient for 50 µl to be added easily by the machine to each HUVEC well. For an example, see Note 6.

3. Switch on an appropriate microplate reader (e.g. FlexStation 2). Open the relevant software (e.g. FlexStation Softmax Pro) and create a new document.
4. Set wavelength to allow measurement of the ratio of emission at 510 nm achieved from excitation of Fura-2 AM dye at 340 nm (calcium-bound state) and 380 nm (calcium-free state).
5. Set the run time for the experiment, the number of readings to take and the interval between each reading. Timings may vary dependent upon stimulus being utilised, source of endothelial cell and expected duration of effect. See Note 7 for example.
6. Specify or select the assay plate format and manufacturer used e.g. 96-well Sarstedt. Clear the program and select which wells in the plate should be read by the instrument.
7. Specify or select the compound source plate format e.g. 96-well U bottom.
8. Input the number, volume and timing of transfers from the compound source (see Note 8).
9. Select which tips to use. Select which column of tips in the rack e.g. column 1, 2, 3 should go into which column of the compound plate e.g. A-H; 1, 2, 3 and then into which column of the cell plate containing HUVECs e.g. A-H; 1, 2, 3.
10. Load the tips, compound plate and cell plate into the microplate reader. Tips and plates should be loaded with A1 situated at the top left.
11. Initiate the read and once started, click display > reduce > plot to see the graphs being produced in real time.
12. Raw data for change in cytosolic calcium levels over time can be exported and analysed in data analysis software e.g. OriginPro (OriginLab, US). The data can be expressed as a raw trace displaying the relative peak height and duration of elevation or can be quantified by calculating the peak magnitude, time taken to reach peak magnitude and area under the curve (figure 1).

**3.4 Immunofluorescence analysis of endothelial NFATc2 localization.**

1. Grow HUVECs in sterile tissue culture grade 96-well plates coated with 100 µl per well porcine skin gelatin as described in 3.1.1.
2. Aspirate medium and starve the cells for 3 h at 37 °C in 90 µl of serum starvation medium.
3. Prepare 10x concentrated VEGF-A stock solutions (0, 2.5, 7.5, 12.5 nM). Make sufficient to allow the addition of 10 µl per well.
4. Add 10 µl of each stock to each well for a final working concentration of 0.25, 0.75 or 1.25 nM. Incubate for 15 min at 37 °C as stimulation period.
5. Aspirate media and fix immediately with 100 µl of pre-warmed fixative followed by incubation for 20 min at room temperature.
6. Wash each well three times with 100 µl of PBS.
7. Permeabilize the cells with fresh permeabilization buffer for 4 minutes at room temperature.
8. Aspirate and wash each well three times with 100 µl of PBS.
9. Block non-specific binding sites by incubation for 1 h at room temperature with 100 µl blocking buffer.
10. Aspirate blocking buffer and add 20 µl/well of 1 µg/ml primary antibody (rabbit anti-NFATc2) in PBS/BSA (see Note 9). Incubate overnight (16-20 h) at 4 °C in a humidified chamber to prevent drying out.
11. The next day, aspirate the primary antibody and wash the wells three times with 100 µl / well of blocking buffer.
12. Prepare secondary antibody by diluting AlexaFluor594-conjugated secondary anti-rabbit antibody to 4 µg/ml in 1 % BSA/PBS; also dilute DAPI to 2 µg/mL from stock solution.

13. Add 20 µl/well of solution containing secondary antibody and DAPI and incubate protected from light at room temperature for 2 h.
14. Wash the cells twice each with 100 µl of blocking buffer followed by one final wash with 100 µl double distilled water.
15. Acquire nuclear DNA (blue) and NFATc2 (red) images for each field of cells using an inverted digital fluorescence microscope. Capture at least 3 fields per well at 20x magnification (figure 2).
16. Use the Coloc 2 plugin for NIH ImageJ software to assess nuclear co-localization by determining overlap of DAPI and NFATc2 staining patterns in the blue and red channels respectively (see Note 10).
17. Open ImageJ and load image. Split channels and retain as separate images the blue and red channels.
18. Select Analyse > Colocalization > Coloc 2. Ensure Costes method is selected for threshold regression to automatically set thresholds without user bias.
19. Choose appropriate algorithms for analysis (see Note 11).
20. Set Costes randomisations to 100.
21. Run the co-localization analysis and note the values for Manders' tM1, Manders' tM2 and Costes p-value (see Note 12).

## 4 Notes

1. HUVECs can be obtained from single donor or pooled mixed donor sources from commercial providers. Cells can also be isolated in-house from single donors as described previously [16,17].
2. Can be prepared in-house as required. Add the necessary amount of pig skin gelatin powder to sterilized PBS and microwave for 1 min until fully dissolved. Push sterilize through a 0.22 µm syringe filter unit in a clean tissue culture laminar flow hood.
3. 3% PFA solution can be prepared in advance by dissolving into PBS. Once fully mixed, CaCl2 and MgCl2 are both added to 0.1 mM before push sterilizing through a 0.22 µm syringe unit. 3% PFA can be stored long-term as aliquots at -20 °C until use. Must be thawed and warmed up to room temperature before use.
4. HUVEC growth rate is variable between batches and dependent upon initial seeding density. Media changes must be performed every 48 h to maintain optimal cell health and growth until they are ready to be used or passaged (at 80 % confluency). Inspect HUVEC growth under the microscope - if confluency is less than 80 %, replace existing media with fresh ECGM and continue to incubate at 37 °C.
5. HUVECs are inherently limited in the number of passages that can be performed. If treated optimally, HUVECs may be maintained until passage 5 without discernible changes in morphology, growth rate or cellular responses.
6. The working concentration of a VEGF-A stimulus is 25 ng/mL, and this therefore requires a 5x stock solution (in SBS) of 125 ng/mL prepared in the U-bottom multiwell plate.
7. For VEGF-A-induced calcium response in HUVECs, set the total run time to 600 sec, and set interval of measurements to every 5 sec. This should equate to a total of 121 readings that are collected during the course of a single run.

8. For a single stimulation with VEGF-A, the programmable on-board pipettor should be set to: 1 transfer of 50 µl from compound source at 32 sec.
9. The primary antibody must be prepared fresh in a solution of 0.1 % (w/v) BSA in PBS immediately before the end of the blocking step.
10. With DAPI as a stain present only in the nucleus, any significant co-localization between the channels is indicative of the nuclear presence of the protein from the red channel.
11. A large number of options are available and may have value depending upon the nature of the experiment. For the purposes of determining the nuclear localization of NFATc2 in HUVECs, select Mander's Correlation and Costes significance test. Further in-depth information on co-localization methods has been published previously[18].
12. If you select the red channel as Channel 1 and blue channel as Channel 2 in the analysis settings, then the tM1 value indicates the proportion of the red pixels that co-localize with blue pixels. The tM2 value indicates the reverse (proportion of blue pixels that co-localize with red pixels). As there can be red signal (NFATc2 not translocated to the nucleus) outside of the nucleus but blue DAPI signal is restricted to the nucleus, these values will be different. The Costes p-value indicates the confidence that the localization is real and should be greater than 0.95.

**Figure 1: VEGF-A stimulation of endothelial cells prompts a significant elevation in cytosolic calcium ions.** Data is shown for control (PBS) vs VEGF-A delivered to HUVECs at 3 different concentrations. (A) Representative output from real-time monitoring of cytosolic calcium ion flux. (B) Quantification of relative peak magnitude provides an indicator of how much calcium ion levels rose after stimulation. (C) Time to peak magnitude graph represents how rapidly the intracellular calcium ion levels rise after stimulation. (D) The graph of relative curve area provides a single quantitative assessment of the level of calcium ion rise and duration for which the elevation is sustained.

**Figure 2: NFATc2 redistribution to the cell nucleus following VEGF-A stimulation of endothelial cells.** (A) Control or non-stimulated cells display a diffuse cytosolic pattern of NFATc2 staining (red) compared to the much more intense nuclear staining of NFATc2 (red) after VEGF-A stimulation. Nuclear DNA stained using DAPI (blue). (B) Quantification of nuclear co-distribution of NFATc2 determined using NIH ImageJ which can be plotted to demonstrate the redistribution of NFATc2 upon VEGF-A stimulation.

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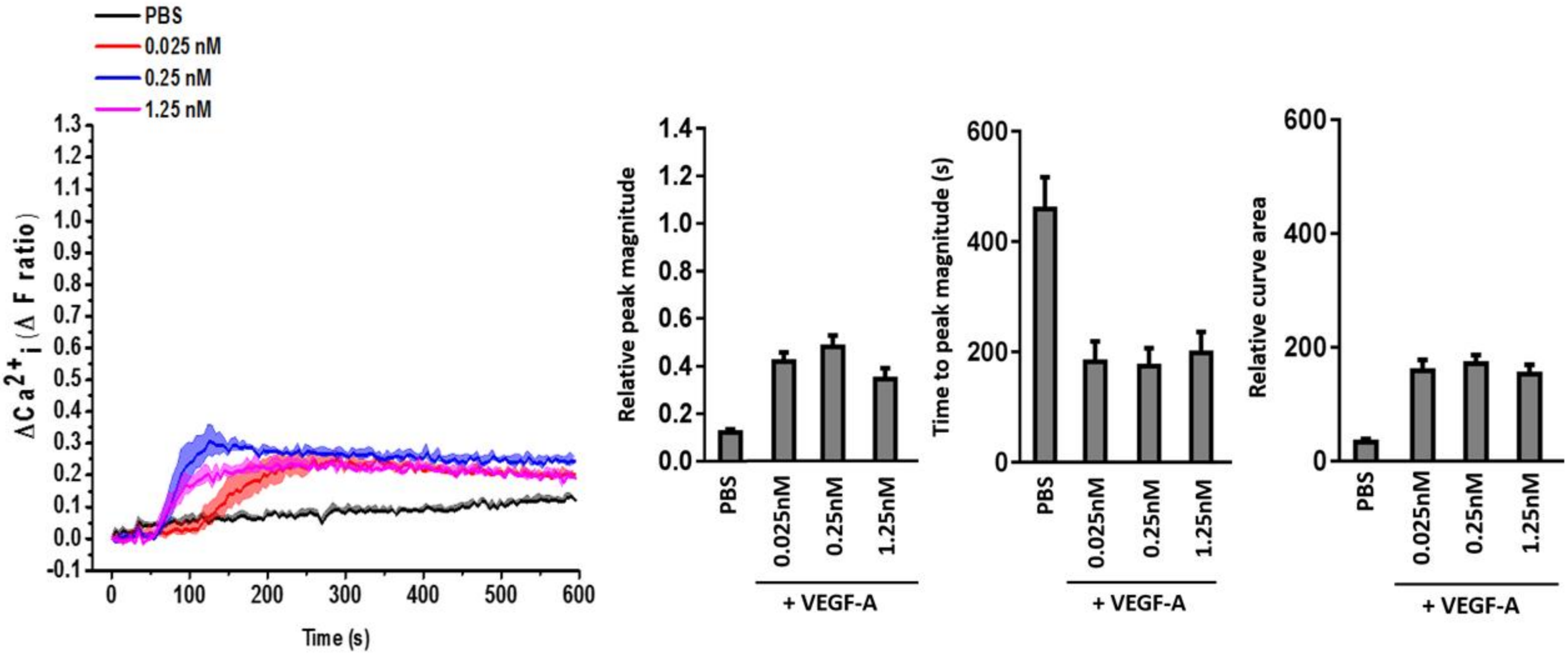
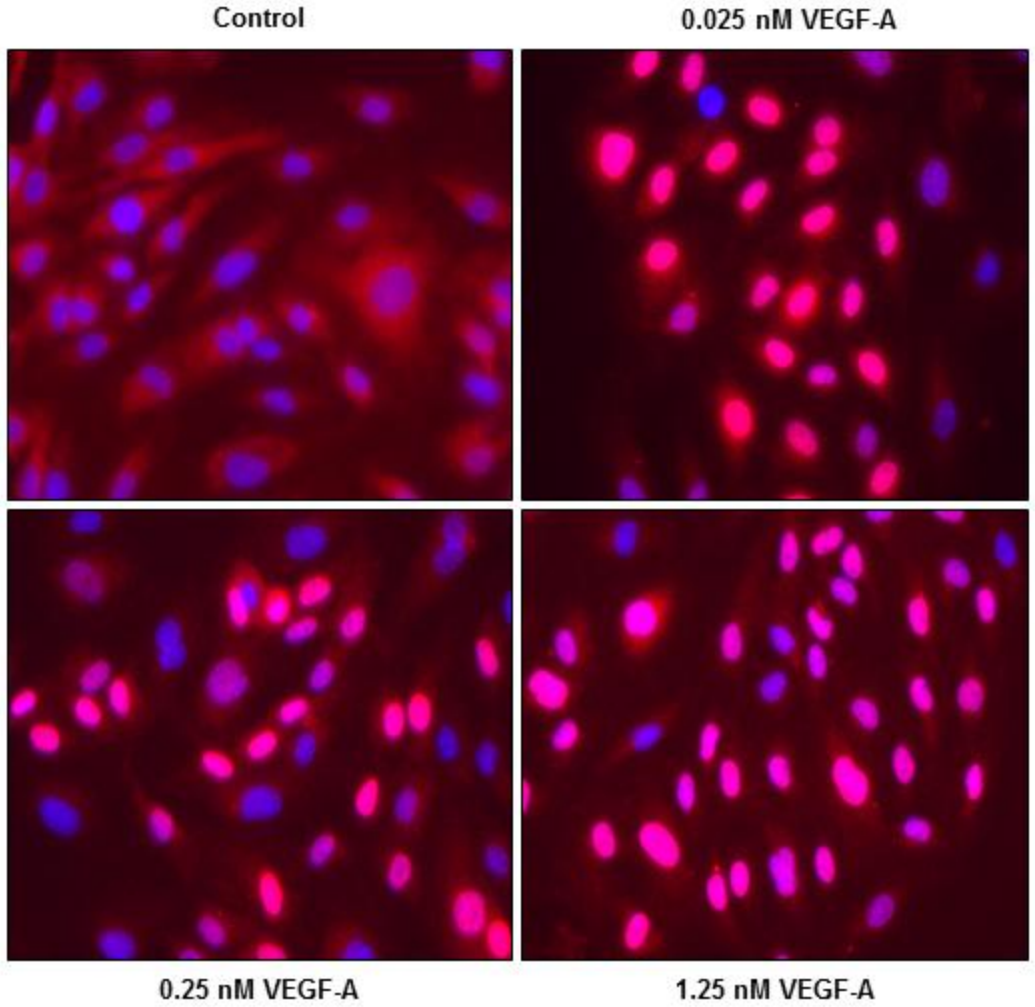

PBS
0.025 nM
0.25 nM
1.25 nM
ΔCa2+i (Δ F ratio)
Time (s)
Relative peak magnitude
Time to peak magnitude (s)
Relative curve area
PBS
0.025nM
0.25nM
1.25nM
+ VEGF-A


Control
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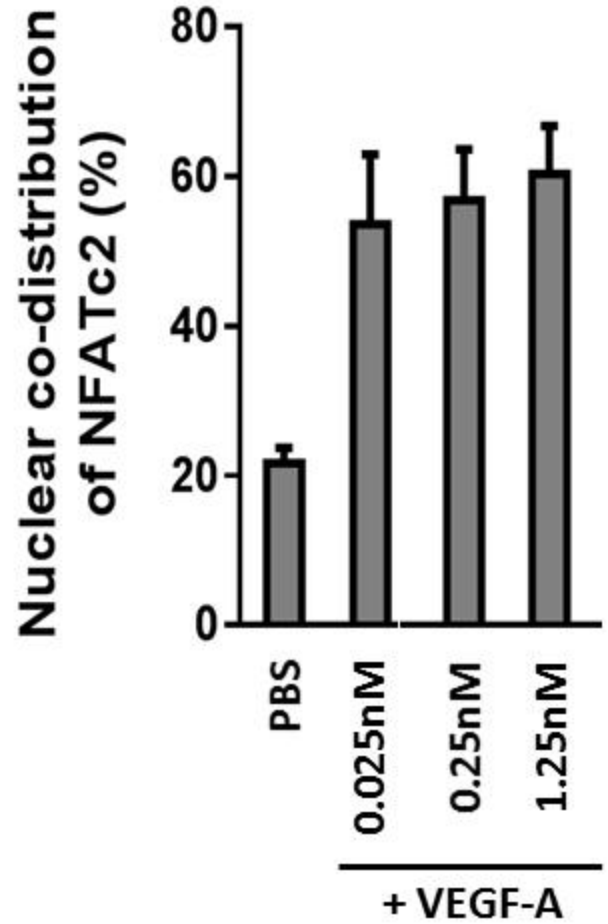
0.25 nM VEGF-A
1.25 nM VEGF-A


Nuclear co-distribution of NFATc2 (%)
80
60
40
20
0
PBS
0.025nM
0.25nM
1.25nM
+ VEGF-A