FRET Imaging of Microenvironment Effects on Calcium Signaling in Live Cells

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<th>Journal:</th>
<th>Integrative Biology</th>
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<tr>
<td>Manuscript ID:</td>
<td>IB-ART-11-2012-020264</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Technical Innovation</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>04-Nov-2012</td>
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</table>
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Insight, Innovation, and Integration
Our results revealed that constrained cell areas resulted from micro-patterns can alter the dynamical characteristics of ATP-induced calcium responses visualized by molecular biosensors based on fluorescence resonance energy transfer (FRET) in single cells and connected neighboring cells. This micro-pattern effect is regulated by voltage operated channels (VOCs) on the plasma membrane. Hence, through the integration of FRET molecular imaging in live cells and micro-patterning technologies, we can significantly advance our mechanistic understanding on how cells perceive micro-environmental cues to regulate intracellular signaling cascades and cellular functions.
FRET Imaging of Calcium Signaling in Live Cells under Microenvironment

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Keywords: Micro/nano-fabrication, Surface pattern, FRET, soft lithography, live cell
imaging

The total number of words of the manuscript, including entire text from title page to figure legends: 4849
The number of words of the abstract: 247
The number of figures: 6
Abstract

Microenvironment has been shown to regulate cellular functions including cell growth, differentiation, proliferation, migration, cancer development and metastasis. However, the underlying molecular mechanism remains largely unclear. We have integrated micro-pattern technology and molecular biosensors based on fluorescence resonance energy transfer (FRET) to visualize calcium responses in cells constrained to grow on micro-patterned surface. Upon ATP stimulation, human umbilical vein endothelial cells (HUVECs) cultured on different surface micro-patterns had a shorter decay time and reduced peak of a transient intracellular calcium rise comparing to control cells without constrains. The decay time is regulated by the plasma membrane and the membrane calcium channels, while the peak by endoplasmic reticulum (ER) calcium release. Further results revealed that voltage operated channels (VOCs), coupling the plasma membrane and ER, can affect both the decay time and the peak of calcium response. The inhibition of VOCs can eliminate the effect of different micro-patterns on calcium signals. When two connected HUVECs were constrained to grow on a micro-pattern, drastically distinct calcium responses upon ATP stimulation can be observed, in contrast to the similar responses of two connected cells cultured without patterns. Interestingly, the inhibition of VOCs also blocked this difference of calcium responses between two connected cells on micro-patterns. These results indicate that micro-patterned surface can have profound effect on the calcium responses of HUVECs under ATP stimulation, largely mediated by VOCs.
Therefore, our results shed new lights on the molecular mechanism by which HUVECs perceive the microenvironment and regulate intracellular calcium signals.
**Introduction**

Microenvironment is an important factor which can affect cellular functions. Indeed, a variety of studies have demonstrated the relationship between microenvironment and the pathophysiological consequences of cells, such as apoptosis (1, 2), cell differentiation (2-5), cell growth and proliferation (6), and cell migration (7-10). Microenvironment has also been shown to affect cancer cells (11). Therefore, the elucidation of microenvironment effect on cells is helpful not only for our understanding of molecular mechanism governing cellular functions, but also for drug development to overcome adverse environmental factors of various diseases (12).

Calcium as a second messenger plays a crucial role in regulating various cellular functions. Indeed, the level of intracellular Ca$^{2+}$ has been shown to regulate mitochondrial functions (13), cell movement (14), cell injury and cell death (15, 16). Intracellular calcium levels can be modulated by the calcium release from ER calcium store and/or calcium influx from extracellular space through membrane calcium channels, such as stretch activated cation channel (SACC), store-operated channel (SOC), and voltage-operated channel (VOC). ATP is well established to regulate intracellular calcium levels by G protein-coupled receptors and PLC-dependent signaling pathways (17-20). There are two phases for intracellular calcium response after ATP stimulation (Figure S1). The first phase is the rising phase of intracellular calcium elevation right after the ATP treatment, which is dependent on the ER calcium release (21). The second phase is the decay phase represented by a decrease
in the intracellular calcium level, which is dependent on the plasma membrane
channels (22). However, it is not clear how microenvironment can affect this
regulation of intracellular calcium signaling cascade stimulated by ATP.

Fluorescence resonance energy transfer (FRET) is a powerful tool to study the
interactions between two molecules within a 10 nm distance. Genetically encoded
biosensors have been developed to monitor various cellular events in live cells (23).
Various versions of FRET biosensors have also been developed to visualize the
intracellular calcium changes in different cells (24, 25). In this paper, we utilized
comb polymer to create micro-patterns on glass surface to control cell
microenvironment, and applied a highly-sensitive calcium FRET biosensor (Figure S2)
to visualize the intracellular calcium signals. We identified VOCs on the plasma
membrane as an important mediator by which the microenvironment affects
intracellular calcium upon ATP stimulation.

**Experimental section**

**Preparation of micro-patterns**

PDMS molds were developed based on silicon masters created by
photolithography as previously described (26). PDMS molds were subsequently
treated with oxygen plasma at the power of 100 W for 30 sec to develop a hydrophilic
surface, rinsed with a 60 mg/ml comb polymer solution in 8:2 (vol./vol.) mixture of
ethanol and deionized water for 10 sec. The molds were spun cast at the speed of
3000 rpm for 15 sec, and the micro patterns were obtained by micro contact printing
(μCP) from the PDMS molds. The glass surfaces with micro-patterns were then
exposed to 30 min UV light for sterilization and subsequently incubated for 4 hr at 37°C with 20 µg/ml Fn for surface coating.

**Polyacrylamide gel for cell culture**

Polyacrylamide gel solutions were prepared from 40% w/v acrylamide stock solution (5%; Bio-Rad) and 2% w/v bis-acrylamide stock solution (0.03-0.3%; Bio-Rad). To polymerize the solutions, 10% w/v ammonium persulfate (Bio-Rad) and N,N,N9,N9-Tetramethylethylenediamine (TEMED; Bio-Rad) were mixed with distilled water. Sulfo-SANPAH [sulfosuccinimidyl6(4_-azide-2_-nitrophenyl-amino) hexanoate; Pierce] was applied to crosslink extracellular matrix molecules onto the gel surface. A detailed protocol about polyacrylamide gels was described previously (27).

**FRET Biosensor and Virus Preparation**

The construct of FRET-based Ca^{2+} biosensor (ECFP-CaM-M13-EYFP) has been described (28). We have replaced EYFP with a recently developed YFP variant YPet to enhance the dynamic range of the biosensor. The fragment containing ECFP, CaM, and M13 was fused to YPet and subcloned into pcDNA3.1 for mammalian cell expression by using BamHI/EcoRI sites. This FRET Ca^{2+} biosensor based on ECFP and YPet allows the visualization and monitoring of intracellular Ca^{2+} signaling with high sensitivity and spatiotemporal resolution (Figure S2). To improve the delivery efficiency of the biosensor into mammalian cells, the DNA sequence encoding the biosensor was further incorporated into an adenoviral vector (Adeno-X Expression System 1, Clontech). The adenovirus carrying the calcium FRET biosensor was then
used to infect HEK cells with 50 percent confluency in the large 100-mm petri dishes (diameter: 100 mm) at the concentration 50 pfu/cell from a high titer stock and then incubated the cells at 37°C in a humidified atmosphere maintained at 5% CO2. After > 50% cells were detached from the surface, confluent HEK cells were collected and transferred to a 15-ml conical centrifuge tube. The suspension was centrifuged at 1500g for 5 min to remove the supernatant and collect the cell pellet. After that, the pellet was re-suspended with 500 μl sterile PBS. The cells were lysed by multiple freezing-thawing cycles: freezing in a dry ice/ethanol bath while thawing in a 37°C water bath for three times. The pellet debris was then centrifuged briefly and the lysate was transferred to and stored in a clean, sterile tube for future usage.

**Cell culture and infection**

Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 (Invitrogen) supplemented with 25% Endothelial Cell Growth Medium (ECGM) (Cell Application) and 20% fetal bovine serum (FBS). The cells were passed to a culture dish 15 hr before infection with adenovirus carrying a calcium FRET biosensor based on ECFP and YPet at the concentration of 50 pfu/cell (29). The cells were passed into glass bottom dishes with micro patterns after 24 hr infection and starved with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% fetal bovine serum (FBS) for 16–24 hr before imaging.

**Microscopy, image acquisition, and analysis**

During imaging, the cells were maintained in 0.5% FBS CO2-independent medium (Invitrogen) at 37 °C. Images were collected by a Zeiss Axiovert microscope
equipped with a cooled CCD camera and controlled by MetaFluor 6.2 software with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters controlled by a filter changer (475DF40 for ECFP and 535DF25 for YPet). The emission intensities of ECFP and YPet were denoted by I(ECFP) and I(YPet), respectively. The pixel-by-pixel ratio images of I(YPet)/I(ECFP) were calculated based on the background-subtracted fluorescence intensity images of ECFP and YPet by the MetaFluor program to allow the quantification and statistical analysis of FRET.

Max ratio time point was picked according to maximal I(YPet)/I(ECFP) value. Decay time was calculated from the Max time point to that where the ratio value returned to the basal level before ATP stimulation.

**Statistical Analysis**

All the ratio data were normalized by their basal levels before stimulation in the same cell. The maximal I(YPet)/I(ECFP) value of the calcium response upon ATP stimulation in each cell was assigned as the Max Ratio for that particular cell. The starting point for the calculation of calcium decay time for each cell was chosen when the calcium response reached the Max Ratio. The ending point of the calcium decay time was determined when the FRET ratio returned to the basal level before ATP stimulation. If the ratio did not return to the basal level by the end of the experiment, the last time point of the experiment was set as the ending point. The decay time was calculated as the difference of the ending and starting points. The decay times under each treatment were graphically represented as a boxplot, where outliers were represented as small circles.
To reduce the estimation bias due to outliers, we estimate the mean decay curve and the 95% confidence bands under each treatment using the robust curve fitting method LOESS (30) implemented in R statistical computing environment. Statistical analysis was also performed by using a Student’s t-test implemented in R to evaluate the statistical difference between groups. A significant difference was determined by the p-value (< 0.05).

Results

To obtain different shapes of micro-patterns coated with extracellular matrix protein fibronectin (Fn) on cover-glass surfaces, we applied micro contact printing (µCP) technique utilizing comb polymer non-adhesive to proteins and hence preventing cell adhesion (31). The created micro-patterns as well as the cells seeded and constrained on the Fn-coated patterns are shown in Figure S3. We have previously engineered a highly sensitive calcium FRET biosensor based on ECFP and YPet pair (29). An adenovirus version of this biosensor was applied to infect human umbilical vein endothelial cells (HUVECs) which were then seeded on the micro-patterned Fn surfaces and subjected to ATP stimulation.

In non-patterned control HUVECs, ATP induced a typical two-phase response of intracellular calcium (Fig. 1A, upper panels). When HUVECs were pretreated with 2-Aminoethoxydiphenyl borate (2APB, 100 mM) to block IP₃ receptor and calcium release from ER, this ATP-induced calcium response was abolished (Fig. 1A, middle panels). The pretreatment by EGTA (5 mM), which chelates and depletes the extracellular calcium in the medium, did not have significant effect on the peak of
ATP-induced calcium response (Fig. 1A, bottom panels). These results confirmed that the ATP-induced calcium response of non-patterned HUVECs is mainly dependent on the ER calcium release, but not the calcium influx from the extracellular medium.

Similar results were observed when HUVECs were cultured on patterned Fn circles (radius=20 µm) (Fig. 1B).

However, the decay phase and hence overall duration of calcium response of the cells on patterned surface appeared shorter than those from non-patterns cells. Further quantitative analysis employing statistical methods revealed that the decay time of the calcium signals in cells cultured on patterned circles (radius =20 µm, area≈1200 µm²) and patterned rectangles (type I rectangles: 20 µm*40 µm, area≈800 µm²), but not other patterned rectangles (type II rectangles: 10 µm*120 µm, area≈1200 µm²), had significant difference from that of non-patterned cells (Fig. 2A, left). Similar phenomena were observed when HUVECs were cultured on polyacrylamide gels with different stiffness (Fig. 2A, right). Indeed, the decay time of cells on soft gels (stiffness=1 Kpa, 5 Kpa, or 10 Kpa), but not on a stiff substrate (stiffness=40 Kpa), was significant reduced comparing to that of non-patterned cells seeded on glass surfaces. The maximal elevation of intracellular calcium concentrations represented by the maximal ratio values of the FRET biosensor (Max Ratio) in cells seeded on the patterned circles or type I rectangles, but not on type II rectangles, was also dramatically decreased comparing to those of non-patterned cells. Interestingly, the max ratio of cells on gels with different stiffness showed no difference from non-patterned cells cultured on glass surfaces either. The maximum
ratio of the calcium response triggered by ATP is dependent on the calcium release from the ER store while the decay phase is mainly determined by the calcium channels on the plasma membrane, such as the store-operated calcium channels (SOC), sodium-associated calcium channel (SACC), and voltage-operated calcium channel (VOC) (32, 33). Therefore, these results suggest that certain micro-patterned environment may affect both calcium release from ER and calcium exchange through the plasma membrane channels. In contrast, gels with different stiffness may affect cell membrane channels but not ER calcium release.

The roles of calcium release from ER and calcium exchange via the plasma membrane in our system were further examined and confirmed by different inhibitors. As shown in figure 3A, non-patterned cells treated with different reagents to manipulate the plasma membrane and channels, including EGTA (5 mM to deplete the extracellular calcium), streptomycin (200 μM to inhibit SACCs), GdCl₃ (10 μM to inhibit SOCs), nifedipine (10 μM to inhibit VOCs), or cholesterol (0.1 M to alter the membrane fluidity) had significantly reduced decay time when compared to the control non-treated cells. Interestingly, only nifedipine among all these reagents decreased the max ratio of the calcium response (Fig. 3B). The time courses of the decay curves were shown in Figure S4 to provide the dynamic information. When non-patterned cells were treated with cytochalasin D (2 μM to disrupt cytoskeletal actin filaments), nocodazole (10 μM to disrupt microtubules), ML-7 (5 μM to inhibit myosin light chain kinase MLCK and hence actomyosin machinery), the results showed that none of these reagents affected the decay time although ML-7 and
nocodazole decreased the max ratio (Fig. 4).

These results confirmed that the plasma membrane and its channels, but not cytoskeletal support, regulate the decay time. The max ratio, on the other hand, only depends on the actomyosin contractility and VOCs which physically couple ER and the plasma membrane (34). Since some micro-patterned surfaces also affected both the decay time and max ratio of calcium response upon ATP stimulation, we then examined whether these effects were mediated by VOCs. As shown in Figure 5, nifedipine eliminated the difference between patterned and non-patterned cells, both in decay time and max ratio of the ATP-induced calcium response. These results suggest that VOCs play a crucial role in mediating the microenvironment effect on the ATP-induced calcium responses.

We further examined the microenvironment effect on the calcium responses of multiple connected cells. As shown in Figure S5 (right), micro-patterns can be created to accommodate two connecting cells in each patterned circle (r=50 μm). When two connected cells were cultured on non-patterned glass surface (Fig. S5 left), ATP caused a similar calcium response of the two cells, including max ratio and decay time (Fig. 6A). Interestingly, two connected cells seeded in the patterned circles had drastically different calcium responses upon ATP stimulation, both in the decay time and max ratio (Fig. 6B). 2APB can completely block these ATP-induced calcium responses of patterned cells whereas EGTA pretreatment eliminated the difference in decay time but not the max ratio (Fig. 6C-D). These results suggest that microenvironment for two connected cells affect both the ER calcium release
reflected by the max ratio and the calcium influx from the extracellular space represented by the decay time. When the two connected cells cultured in patterned circles were pretreated with nifedipine, no more differences in the decay time and max ratio between these two cells were observed (Fig. 6E-F). These results suggest that VOCs, by coupling ER and the plasma membrane, mediate the microenvironment effect on the calcium responses from connected multiple cells.

**Discussion**

Microenvironment is crucial to cellular functions. Here we applied micro-fabrication technology to create Fn patterns on glass surface such that cells can be constrained to grow on these patterns for the investigation of microenvironment effects on intracellular molecular signals in regulating cellular functions. Our results revealed that micro-patterned surface can affect both the decay time (mainly mediated by the plasma membrane and its calcium channels) and the max ratio (mainly regulated by the calcium release from ER) of the calcium response in HUVECs upon ATP stimulation. Interestingly, substrates with different mechanical stiffness can only affect the decay time, but not the max ratio. These results indicate that while both micro-patterned surface and substrates with different mechanical stiffness can modulate the microenvironment and ATP-induced calcium response, they differ in the regulation mechanism in that micro-patterned surface affect both the plasma membrane and ER whereas mechanical substrates impact more on the plasma membrane. While the detailed mechanism about this difference is not clear, it is possible that the strict space constrains enforced by the micro-patterns may have more
impact on the ER size (data not shown) and regulate ER functions in calcium release. Indeed, it has been shown that the size rather than the shape of ER is critical in regulating the ER stress and functions (35).

It is intriguing that two connected cells constrained in a micro-patterned surface had drastically different responses in calcium signaling upon ATP stimulation. It is possible that the micro-patterned surface and the limited space enforced an asymmetric constrain on both ER and the plasma membrane of the two neighboring cells. In contrast, two connected cells without patterns have very similar time courses of calcium response upon ATP stimulation, likely reflecting a well adjusted coordination of these two cells in both ER and the plasma membrane.

The slow decay time of calcium response upon ATP stimulation is similarly observed in a previous report indicating a major contribution of the calcium influx from extracellular medium during this phase (36). Consistently, the perturbation of the plasma membrane and the calcium channels on the membrane modulated this decay time. It is apparent that the max ratio of ATP-induced calcium response and hence ER calcium release is relatively independent of general membrane perturbation as none of the membrane perturbation reagents except nifedipine affected the max ratio.

Nifedipine can clearly inhibit both the decay time as well as the max ratio, possibly reflecting its inhibitory role in VOCs which are anchored on the plasma membrane and coupled to ER (34). The difference in calcium response between two connected cells on micro-patterned surface can also be eliminated by nifedipine treatment, which clearly indicates a crucial role of VOCs in mediating the microenvironment effect on
calcium signaling.

The manipulation of cytoskeleton and actomyosin contractility did not affect the decay time, consistent with the note that the decay time of ATP-induced calcium response is mediated by the calcium influx through membrane channels (36). Interestingly, nocodazole or ML-7, corresponding inhibitors of microtubules or myosin light chain kinase (MLCK) (37), decreased the max ratio of ATP-induced calcium response. In contrast, cytochalasin D, an inhibitor of actin filaments (38), did not have significant effect. These results indicate that different cytoskeleton components and actomyosin contractility can have distinctive effect on the ER and subsequently the ER calcium release.

Calcium regulates a variety of endothelial cell functions, including increase of nitric oxide and prostacyclin, which leads to vasodilation. Previous studies suggest that the difference in the peak and duration of [Ca2+], which is strongly dependent on decay phase, contributes to the cell proliferation (22) and the activation of various transcription factors (39, 40). Therefore, our results that micropattern and microenvironment can have substantial impact on the peak and decay characteristics of intracellular calcium signals should shed new light on the molecular mechanism by which microenvironment regulates the endothelial cellular functions and physiology, e.g. vasodilation.

In summary, we have integrated FRET biosensors and micro-pattern technology to investigate the microenvironment effect on the intracellular molecular signals in live cells. The results indicate a clear impact of microenvironment on the calcium
signals upon ATP stimulation, which is mainly mediated by VOCs. The information should advance our insightful understanding of the molecular mechanism by which cells perceive the microenvironment and accordingly coordinate biochemical signals to regulate cellular functions. The integration of micro-patterns and molecular FRET biosensors can also provide powerful platforms for future studies to visualize, in principle, the microenvironment effect on any molecular signal in single live cells with high spatiotemporal resolutions. The combined information obtained should allow the mapping out of the whole molecular network governing the cell physiology under different environment.

References:


Figure Legends:

Figure 1. The calcium responses upon ATP stimulation in HUVECs are mediated by IP3 receptors on ER in both non-patterned and patterned HUVECs.

(A) The time courses and representative images of YPet/ECFP emission ratio of calcium biosensor in non-patterned HUVECs before and after 1 µM ATP stimulation. Upper panels: non-patterned control HUVECs; Middle panels: non-patterned HUVECs pretreated with 100 mM 2APB to block ER IP3 receptors; Lower panels: non-patterned HUVECs pretreated with 5 mM EGTA to remove free calcium in cell culture medium.

(B) The time courses and representative images of YPet/ECFP emission ratio of calcium biosensor in HUVECs constrained to grow on micro-patterns (Circle, r=20 µm) before and after 1 µM ATP stimulation under different conditions as shown in (A).

The color bars represent the levels of YPet/ECFP emission ratios, with the cold and hot colors representing the low and high ratios, respectively.

Figure 2
Micro-patterns affect both the max ratio and decay time of calcium responses upon ATP stimulation in single HUVECs while soft gel substrates only affect the decay time.

(A) Boxplots represent the decay times of calcium responses upon ATP stimulation in HUVECs. Left: HUVECs were cultured on glass surface without patterns (Non Pattern), on circles with a radius of 20 µm (Circle r:20), on rectangles with sizes 20 µm X 40 µm (Rec 20X40), or on rectangles with sizes 10 µm X 120 µm (Rec 10X120). Right: HUVECs were cultured on glass surface without patterns (Non Pattern) or on different substrates with varying stiffness (1 Kpa, 5 Kpa, 10 Kpa, 40 Kpa as indicated).

(B) Boxplots represent the max ratios of calcium responses upon ATP stimulation in HUVECs under conditions as shown in (A).

The small circles “•” represent outliers from each group.

+ represents a statistical P <0.05 when comparing to the non-patterned group on glass surface.

Figure 3
The effect of reagents perturbing the plasma membrane and membrane channels.

(A) Boxplots represent the decay time of calcium responses upon ATP stimulation in non-patterned HUVECs cultured on glass surface under different conditions: control (Non Pattern), pretreated with 5 mM EGTA for 10 min to remove free extracellular calcium, with 200 µM streptomycin for 30 min to block SACC, with 10 µM GdCl3 for 30 min to block SOC, with 10 µM nifedipine for 30 min to block VOCs, or with 0.1M cholesterol for 3 hr to alter the membrane fluidity.

(B) Barplots represent the max ratio of calcium responses upon ATP stimulation in HUVECs under conditions as shown in (A).
+ represents a statistical P < 0.05 when comparing to the non-patterned group on glass surface.
The small circles “◆” represent outliers from each group.

**Figure 4**
VOCs, but not cytoskeleton or actomyosin contractility, play crucial roles in regulating both the decay time and max ratio of calcium responses upon ATP stimulation.

**A** Boxplots represent the decay time of calcium responses upon ATP stimulation in non-patterned HUVECs cultured on glass surface under different conditions: control (Non Pattern), pretreated with 10 μM nifedipine (10 μM) for 30 min to block VOCs, with 2 μM cytochalasin D for 30 min to disrupt actin filaments, with 10 μM nocodazole for 30 min to disrupt microtubules, or with 5 μM ML-7 for 30 min to inhibit actomyosin contractility.

**B** Boxplots represent the max ratio of calcium responses upon ATP stimulation in HUVECs under conditions as shown in (A).

+ represents a statistical P < 0.05 when comparing to the non-patterned group on glass surface.
The small circles “◆” represent outliers from each group.

**Figure 5**
VOCs play crucial roles in mediating the effect of micropatterns on both the decay time and max ratio of calcium responses upon ATP stimulation.

**A** Boxplots represent the decay time of calcium responses upon ATP stimulation in HUVECs cultured on glass surface patterns with or without nifedipine treatment as indicated: control (Non Pattern), on circles with a radius of 20 μm (Circle r:20), on rectangles with sizes 20 μm X 40 μm (Rec 20X40), or on rectangles with sizes 10 μm X 120 μm (Rec 10X120).

**B** Boxplots represent the max ratio of calcium responses upon ATP stimulation in HUVECs under conditions as shown in (A).

+ represents a statistical P < 0.05 when comparing to the non-patterned group on glass surface.
The small circles “◆” represent outliers from each group.

**Figure 6**
VOCs mediate the drastic difference in the decay time and max ratio of calcium responses in two connected HUVECs cultured on micro-patterned circles.

**A** The time courses and representative images of YPet/ECFP emission ratio of calcium biosensor in two connected HUVECs without patterns before and after 1 μM ATP stimulation.

**B-D** The time courses and representative images of YPet/ECFP emission ratio of calcium biosensor in two connected HUVECs cultured on a micro-patterned circle (r=50 μm) before and after 1 μM ATP stimulation. (B): control cells; (C): cells pretreated with 2APB; (D): cells pretreated with
EGTA.

(E) Boxplots represent the difference in decay time of calcium responses upon ATP stimulation in two connected HUVECs cultured without patterns (Non-Pattern), or on a micro-patterned circle(r=50 μm) with (Pattern+Nifedipine) or without nifedipine pretreatment (Pattern).

(F) Boxplots represent the difference in max ratio of calcium responses upon ATP stimulation in two connected HUVECs under conditions as shown in (A).

+ represents a statistical P <0.05 when comparing to the non-patterned group on glass surface. The small circles “·” represent outliers from each group.

The color bars represent the levels of YPet/ECFP emission ratios, with the cold and hot colors representing the low and high ratios, respectively.