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**Rhythmic contraction and intracellular Ca<sup>2+</sup> oscillatory rhythm in spontaneously  
beating cultured cardiac myocytes.**

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**Running head:** rhythmic contraction and Ca<sup>2+</sup> oscillation

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## **Abstract**

Cultured cardiac myocytes from neonatal rats show spontaneous and rhythmic contractions. The intracellular concentration of free  $\text{Ca}^{2+}$  also changes rhythmically associated with the rhythmic contraction of myocytes ( $\text{Ca}^{2+}$  oscillation). This study aims to elucidate whether spontaneous rhythmic contraction affects the dynamics of intracellular  $\text{Ca}^{2+}$  oscillation in cultured cardiac myocytes. In cultures at 4 days *in vitro* (4 DIV), spontaneous  $\text{Ca}^{2+}$  oscillation was synchronized among myocytes. Treatment of cultures with an uncoupler of E-C coupling resulted in a cessation of the spontaneous contraction of cardiac myocytes, but did not abolish the  $\text{Ca}^{2+}$  oscillation. The intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation persisted, and both the intervals and the fluctuation of the oscillation tended to increase after the termination of rhythmic contraction. The present study demonstrated that mechanical factors associated with rhythmic contraction did not affect the intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation, but possibly contributed to the stability of the oscillatory rhythm.

**Key words:**  $\text{Ca}^{2+}$  oscillation, rhythmic contraction, intercellular synchronization, E-C coupling, fluctuation

## 1. Introduction

Isolated and cultured neonatal cardiac myocytes contract spontaneously and cyclically (Harary and Farley 1963). The contraction rhythms of two isolated cardiac myocytes in culture, each of which beats at different frequencies at first, become synchronized after the establishment of mutual contacts (Jongsma *et al.* 1987), suggesting that mutual entrainment occurs due to electrical and/or mechanical interactions between two myocytes. The intracellular concentration of free  $\text{Ca}^{2+}$  also changes rhythmically associated with the rhythmic contraction of myocytes ( $\text{Ca}^{2+}$  oscillation). The  $\text{Ca}^{2+}$  oscillation was also synchronized among cultured cardiac myocytes (Kimura *et al.* 1995).

We have recently demonstrated that the fluctuation of beating rhythm, estimated from the coefficient of variation (CV) of contraction intervals, changes depending on the strength of coupling through gap junctions (GJ) among spontaneously beating cardiac myocytes (Yamauchi *et al.* 2002). The reduced fluctuation of beating rhythm depending on the culture period seems to reflect the synchronization of contraction rhythm among myocytes caused by the increased mechanical and/or electrical coupling strength via GJ. Although the synchronization of intracellular  $\text{Ca}^{2+}$  oscillation among cultured cardiac myocytes has also been primarily attained via GJ intercellular communication (Kimura *et al.* 1995), the exact mechanisms responsible for

the intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation are not yet fully understood.

Previous studies have revealed that cardiac myocytes have mechano-sensitive ion channels such as stretch-activated channels (Kawakubo *et al.* 1999; Zeng *et al.* 2000), indicating the possibility that cyclic mechanical movements affect intracellular  $\text{Ca}^{2+}$  oscillation. Here we investigated whether the cellular dynamics of intracellular  $\text{Ca}^{2+}$  oscillation in cardiac myocytes changed when the contractile activity was suppressed by treatment with 2,3-butanedione monoxime (BDM), a reversible blocker of cardiac contraction (Cheng *et al.* 1997; Gwathmey *et al.* 1991; Kurihara *et al.* 1990). The present study has demonstrated that mechanical factors associated with rhythmic contraction did not affect the intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation, but contributed to the stabilization of the oscillation rhythm in cultured cardiac myocytes.

## 2. Materials and methods

The animal experiments conformed to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996), as well as the "guide for the care and use of laboratory animals", Hokkaido University School of Medicine.

### 2.1 Culture of cardiac myocytes

The method of culture was described elsewhere in detail (Kawahara *et al.*, 2002a; Yamauchi *et al.*, 2002; Kohashi *et al.*, 2003). In short, cardiac myocytes were prepared from ventricles of 1 to 3-day old neonatal Wistar rats removed after decapitation. The ventricles were rinsed in a 25 mM HEPES-buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1% collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37 °C for 10 min. The digested fragments were centrifuged at 1000 rpm for 2 min (LC-100, TOMY, Japan) and precipitated cell components were washed twice with MSS to terminate the effects of collagenase. The cell components were suspended in MCDB 107 (Research Institute for the Functional Peptides, Yamagata, Japan) containing 5% FCS (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90  $\mu$  m porosity) to remove large aggregates of cells; the filtered suspension contains cardiac myocytes and fibroblasts. To separate cardiac myocytes

from fibroblasts based on the selective adhesion technique, the cell suspension was poured into petri dishes ( $\phi$  60 mm, Falcon), and incubated for 60 min at 37 ° C, in 5% CO<sub>2</sub> and 95% air. By virtue of the procedure, most of the fibroblasts adhere to the dish. After the incubation, the suspension, mostly containing cardiac myocytes, was collected. The suspension was centrifuged at 700 rpm for 5 min to separate the remaining blood cell components in the supernatant. The precipitated cells were resuspended in MCDB 107 containing 5% FCS, transferrin (10  $\mu$  g/mL, Sigma, St. Louis, MO), and insulin (10  $\mu$  g/mL, Yamanouchi, Tokyo, Japan). The cell suspension was passed through a fine wire mesh screen (25  $\mu$  m porosity) to remove remaining small aggregates of myocytes, and finally the isolated myocytes remaining were cultured at a density of about  $3.0 \times 10^5$  cells/ml in a petri dish ( $\phi$  30 mm, Falcon) coated beforehand with fibronectine (10  $\mu$  g/mL, Sigma). Cardiac myocytes cultured for 4 days were used in this study.

## 2.2 Image analysis

In this study, the spontaneous contraction rhythm of cultured myocytes was evaluated using a video image recording method. This procedure is described elsewhere in detail (Kawahara *et al.*, 2002a; Kohashi *et al.*, 2003; Yamauchi *et al.*, 2002; Yoneyama and Kawahara, 2004). In short, images of beating myocytes were recorded with a CCD camera (WV-BD400, Panasonic,

Japan) through a phase-contrast microscope (IX70, OLYMPUS, Japan). A spontaneously beating myocyte was arbitrarily selected from myocytes in the video image. A small area (a square of about 20 pixels) of the myocyte where brightness changed considerably with contraction was selected, and the video signals were digitized to an 8-bit number every video frame (30 frames/s) by a video capture board in a personal computer (Power Macintosh 7500/100, Apple).

A reference frame was arbitrarily selected and cross-correlograms were calculated between pixels of the reference frame and those of other frames, to represent the temporal variation of brightness in the selected area corresponding to the contraction rhythm of the cardiac myocyte.

### *2.3 Cellular $Ca^{2+}$ measurements*

Changes in cytosolic free  $Ca^{2+}$  were measured using fluo 4. Cardiac myocytes in culture were loaded with the fluorescent calcium indicator during a 30 min incubation with acetoxymethyl ester of fluo 4 (fluo 4/AM, 5  $\mu$  M; Molecular probes, Eugene, OR) in MCDB medium at room temperature. Fluo 4 was excited at 490 nm, and emission intensity was measured at 525 nm. Fluorescent images were acquired at about 200 msec intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu, Japan). An analysis of

the acquired images was done with an image processing and measuring system (AQUACOSMOS; Hamamatsu Photonics). Fluorescent intensity ( $F$ ) was normalized with the initial value ( $F_0$ ), and the changes in the relative fluorescent intensity ( $F/F_0 - 1$ ) were used to assess those in cellular free  $Ca^{2+}$ .

#### *2.4 Experimental protocol*

We first confirmed that cultured cardiac myocyte after 4 days in vitro (4 DIV) exhibited spontaneous beating. To terminate the spontaneous rhythmic contraction of cardiac myocytes, 2,3-butanedione monoxime (BDM) was loaded into cells. BDM has been found to reversibly block cardiac contraction, without blocking electrical activities (Cheng et al. 1997). BDM was added to MCDB, and the final concentration in MCDB was adjusted to 7.5 mM. This concentration of BDM almost completely terminated spontaneous contraction, but did not produce detectable changes in the peak-to-peak amplitude of  $Ca^{2+}$  oscillation in cardiac myocytes (see Figs. 2-4). The loading of cells with a concentration of BDM greater than 10 mM resulted in a decrease in the peak-to-peak amplitude of  $Ca^{2+}$  oscillation, and in some cases, terminated  $Ca^{2+}$  oscillation as well as cell contraction (data not shown).

#### *2.5 Chemicals*

Verapamil and 2,3-butanedione monoxime (BDM) were obtained from Sigma. Fluo-4/AM was obtained from Molecular probes (Eugene, OR). The other chemicals were from Wako Chem.

### *2.6 Statistical analysis*

The data are expressed as the mean  $\pm$  S.D. Comparisons were performed using a paired t-Test. A *P* value of less than 0.05 was considered significant.

### 3. Results

#### 3.1 Intercellular synchronization of intracellular $Ca^{2+}$ oscillation

Cyclic changes in the concentration of intracellular free  $Ca^{2+}$  ( $Ca^{2+}$  oscillation) in cultured cardiac myocytes were measured by loading myocytes with fluo 4/AM. Isolated and cultured neonatal cardiac myocytes started to contract spontaneously and cyclically after 2-to-4 days *in vitro* (2-4 DIV). The intracellular concentration of free  $Ca^{2+}$  also changed cyclically associated with the spontaneous rhythmic contraction of cardiac myocytes. We first investigated whether the spontaneous cyclic changes in the concentration of intracellular free  $Ca^{2+}$  in cultured cardiac myocytes were synchronized among myocytes using a fluorescent  $Ca^{2+}$  indicator, fluo 4/AM (Fig. 1). The relative intensity of fluo 4 fluorescence in three cells (Fig. 1A2), the same cells as indicated in Fig. 1A1, fluctuated cyclically and the  $Ca^{2+}$  oscillation was synchronized among myocytes (Fig. 1B1-B3). In all of the cultures tested, the  $Ca^{2+}$  oscillation in cardiac myocytes was synchronized among the cells.

#### 3.2 Spontaneous rhythmic contraction and $Ca^{2+}$ oscillation

We next investigated whether the cyclic changes in the intracellular concentration of free  $Ca^{2+}$  ( $Ca^{2+}$  oscillation) persisted after the termination of spontaneous rhythmic contraction. The contractile activity of cardiac myocytes was suppressed by treatment with 2,3-butanedione

monoxime (BDM), a reversible blocker of cardiac contraction (Cheng *et al.* 1997; Gwathmey *et al.* 1991; Kurihara *et al.* 1990). Loading of cultured cardiac myocytes with 7.5 mM BDM for 20 min resulted in the termination of spontaneous contraction (Fig. 2B2), but  $\text{Ca}^{2+}$  oscillation was still observed (Fig. 2C2).

### *3.3 Rhythmic contraction and intercellular synchronization of $\text{Ca}^{2+}$ oscillation*

Previous studies have demonstrated that cardiac myocytes have mechano-sensitive ion channels such as stretch-activated channels (Kawakubo *et al.* 1999; Zeng *et al.* 2000), indicating the possibility that cyclic mechanical movements associated with the rhythmic contraction of myocytes would affect the intercellular synchronization of the intracellular concentration of  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  oscillation). Therefore, we then investigated whether the intercellular synchronization of  $\text{Ca}^{2+}$  oscillation in cardiac myocytes changed when the contractile activity was suppressed by treatment with BDM. Loading of cultured cardiac myocytes with 7.5 mM BDM for 20 min resulted in the termination of spontaneous rhythmic contraction (Fig. 3B2), but did not affect the intercellular synchronization of  $\text{Ca}^{2+}$  oscillation at all (Fig. 3C2). Treatment with this concentration of BDM terminated the rhythmic contraction of almost all the myocytes observed.

### *3.4 Rhythmic contraction and the fluctuation of Ca<sup>2+</sup> oscillatory rhythm*

Treatment of cultured cardiac myocytes with 7.5 mM BDM for 20 min resulted in the elongation of the peak-to-peak intervals of Ca<sup>2+</sup> oscillation (Fig. 3C2). In addition, the treatment also tended to increase the fluctuation of the intervals of Ca<sup>2+</sup> oscillatory rhythm (Fig. 3C2). Therefore, we next performed a statistical analysis on the changes in the peak-to-peak intervals and the fluctuation of intervals in the Ca<sup>2+</sup> oscillatory rhythm when the spontaneous rhythmic contraction was terminated by treatment with BDM. Figure 4 shows the changes in the oscillation intervals, the fluctuation assessed with a coefficient of variation (CV), and in the peak-to-peak amplitude of Ca<sup>2+</sup> oscillation. Treatment of cultures with 7.5 mM BDM resulted in a significant increase in the oscillation intervals (Fig. 4A). The treatment also increased the CV of the intervals (Fig. 4B), although the increase was not statistically significant. However, the treatment did not change the peak-to-peak amplitude of Ca<sup>2+</sup> oscillation (Fig. 4C1 and 4C2). In some cases, the treatment increased the peak-to-peak amplitude (Fig. 4C3).

### *3.3 Verapamil-induced termination of spontaneous contraction and Ca<sup>2+</sup> oscillation*

In this study, cardiac myocytes were treated with BDM to terminate spontaneous contraction. Although BDM is well known as an electromechanical uncoupler (Cheng *et al.*, 1997), that is, an uncoupler of excitation-contraction coupling (E-C coupling), a previous study

has suggested that it also inhibits the activity of sarcolemmal L-type  $\text{Ca}^{2+}$  channels (Ferreira *et al.* 1997). This raises the possibility that changes in the intervals of  $\text{Ca}^{2+}$  oscillation observed when spontaneous rhythmic contraction was terminated by treatment with BDM may be caused by the BDM-induced inhibition of the activity of L-type  $\text{Ca}^{2+}$  channels. Therefore, we next investigated whether the dynamics of  $\text{Ca}^{2+}$  oscillation changed when myocytes were treated with verapamil, a blocker of L-type  $\text{Ca}^{2+}$  channels.

Treatment of cardiac myocytes with 1.0  $\mu\text{M}$  verapamil for 5 min resulted in the reversible termination of spontaneous contraction similar to BDM (Fig. 5B2). In contrast to BDM, however, treatment with verapamil also terminated the spontaneous intracellular  $\text{Ca}^{2+}$  oscillation (Fig. 5C2), suggesting that  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels was necessary for the spontaneous  $\text{Ca}^{2+}$  oscillation in cardiac myocytes, and that changes in the intervals of  $\text{Ca}^{2+}$  oscillation observed when spontaneous rhythmic contraction was terminated by treatment with 7.5 mM BDM were not caused by the BDM-induced inhibition of the activity of L-type  $\text{Ca}^{2+}$  channels.

## Discussion

The present study has demonstrated that  $\text{Ca}^{2+}$  oscillation was still observed when the spontaneous rhythmic contraction was suppressed by the loading of cultured cardiac myocytes with BDM (Fig. 2), and that mechanical cyclic movement did not affect the intercellular synchronization of the intracellular  $\text{Ca}^{2+}$  oscillation (Fig. 3).

In this study, the termination of spontaneous cyclic contraction of cardiac myocytes by treatment with BDM elongated the intervals of intracellular  $\text{Ca}^{2+}$  oscillation (Figs. 3C2 and 4A). Previous studies have demonstrated that BDM not only uncouples the E-C coupling of cardiac myocytes, but also inhibits the activity of sarcolemmal L-type  $\text{Ca}^{2+}$  channels (Byron *et al.* 1996; Dooley *et al.* 1999; Ferreira *et al.* 1997). Thus, it was possible that the elongation of intervals of  $\text{Ca}^{2+}$  oscillation was due to the BDM-induced inhibition of the activity of L-type  $\text{Ca}^{2+}$  channels. However, this possibility seemed unlikely. Treatment of cardiac myocytes with 7.5 mM BDM almost completely terminated the contraction, but did not change the peak-to-peak amplitude of the intracellular  $\text{Ca}^{2+}$  oscillation (Figs. 3C2 and 4C1-C3). In addition, treatment with verapamil, a blocker of L-type  $\text{Ca}^{2+}$  channels, not only terminated the spontaneous contraction of cardiac myocytes, but also abolished the intracellular  $\text{Ca}^{2+}$  oscillation (Fig. 5), suggesting that BDM-induced elongation of the intervals of  $\text{Ca}^{2+}$  oscillation was not caused by the BDM-induced inhibition of the activity of L-type  $\text{Ca}^{2+}$  channels. If this was the case, the

question arises as to what mechanisms are involved in the BDM-induced elongation of the intervals of  $\text{Ca}^{2+}$  oscillation observed in this study. The exact mechanisms are currently unknown, but our computer simulation with mutually interacting Bonhoeffer-van der Pol (BVP) oscillators demonstrated that the termination of spontaneous cyclic movements resulted in the elongation of the intervals of  $\text{Ca}^{2+}$  oscillation (paper in preparation). Thus, the elongation of the intervals of  $\text{Ca}^{2+}$  oscillation induced by terminating spontaneous rhythmic contraction was probably due to a fundamental and intrinsic property of the mutually interacting non-linear oscillators such as spontaneously beating cardiac myocytes.

Treatment of cardiac myocytes with BDM also tended to increase the fluctuation of the intervals of  $\text{Ca}^{2+}$  oscillation estimated using the co-efficient of variation (CV) of the intervals (Figs. 3C2 and 4B). A previous study has reported that treatment of cultured cardiac myocytes with BDM reversibly blocks gap-junctional intercellular communications (Verrecchia and Herve, 1997). We have previously demonstrated that gap junction blockade results in the increased fluctuation of beating intervals in cardiac myocytes (Kawahara *et al.*, 2002). Thus, there is a possibility that the BDM-induced increase in the fluctuation of the intervals of  $\text{Ca}^{2+}$  oscillation was due to the BDM-induced blockade of gap junctions. However, this seems to be an unlikely cause. Treatment with BDM did not affect the intercellular synchronization of the intracellular  $\text{Ca}^{2+}$  oscillation (Fig. 3), suggesting that intercellular communications via gap

junctions or other mechanisms remained functional even after the termination of rhythmic movements by treatment with BDM. Thus, the BDM-induced increase in the fluctuation of  $\text{Ca}^{2+}$  oscillation may be also due to a fundamental and intrinsic property of the mutually interacting non-linear oscillators.

This study demonstrated that the concentration of intracellular free  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  oscillation) was synchronized among cultured cardiac myocytes. The  $\text{Ca}^{2+}$  oscillation was synchronized not only among myocytes in an aggregate, but also among myocytes without physical contact separated from each other (See cell 3 in Fig. 1). Treatment with a blocker of gap junction channels did not affect the synchronization, suggesting that a mechanism other than intercellular communication via gap junctions was involved in the intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation (paper in preparation). The signaling pathway responsible for the intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation in spontaneously beating cultured cardiac myocytes is under investigation.

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## Figure legends

Fig. 1

Intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation in cardiac myocytes in culture. The  $\text{Ca}^{2+}$  oscillation in cultured neonatal cardiac myocytes at 4 days *in vitro* (4 DIV) indicated by arrows in the phase-contrast image of the culture (A1) and in the fluorescence image (A2) was synchronized among myocytes (B1-B3). Cyclic changes in the concentration of intracellular free  $\text{Ca}^{2+}$  were measured by loading myocytes with a fluorescent  $\text{Ca}^{2+}$  probe fluo-4/AM (5  $\mu\text{M}$ ). The scale bar indicates 100  $\mu\text{m}$ , and applies to both A1 and A2.

Fig. 2

Spontaneous rhythmic contraction and intracellular  $\text{Ca}^{2+}$  oscillation in cardiac myocytes in culture. A cultured neonatal cardiac myocyte at 4 DIV indicated by an arrow in the phase-contrast image of the culture (A1) showed spontaneous rhythmic contraction (B1). The same myocyte indicated by an arrow in the fluorescence image (A2) showed spontaneous intracellular  $\text{Ca}^{2+}$  oscillation (C1). Loading of cultured cardiac myocytes with 7.5 mM 2,3-butanedione monoxime (BDM), a reversible blocker of cardiac contraction, for 20 min terminated the spontaneous contraction of cardiac myocytes (B2), but the  $\text{Ca}^{2+}$  oscillation was

still observed (C2). The scale bar indicates 100  $\mu$  m, and applies to both A1 and A2.

Fig. 3

Persistence of intercellular synchronization of intracellular  $\text{Ca}^{2+}$  oscillation in quiescent cultured cardiac myocytes treated with BDM. The spontaneous rhythmic contraction of a cultured cardiac myocyte (cell 3 in A1) was almost completely terminated by the loading of cells with 7.5 mM BDM for 20 min (B2). After the washout of BDM, the rhythmic contraction gradually recovered (B3). The cyclic changes in the concentration of intracellular  $\text{Ca}^{2+}$  in three cardiac myocytes indicated by arrows in the phase-contrast image (A1) and in the fluo 4-fluorescent image (A2) were synchronized before the onset of treatment with BDM (C1), during the treatment (C2), and 20 min after the washing out (C3). It should be noted that treatment with BDM elongated the peak-to-peak interval of  $\text{Ca}^{2+}$  oscillation as compared with that before treatment (C2). The scale bar indicates 100  $\mu$  m, and applies to both A1 and A2.

Fig. 4

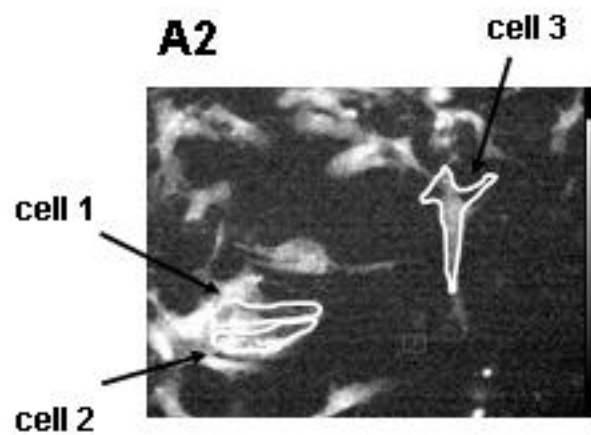
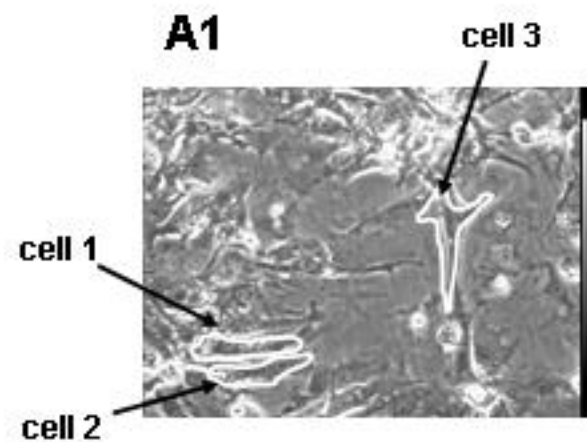
BDM-induced changes in intracellular  $\text{Ca}^{2+}$  oscillation in cultured cardiac myocytes. Cardiac myocytes (4 DIV) were loaded with 7.5 mM BDM for 20 min. Figure A shows the BDM-induced change in the peak-to-peak intervals of  $\text{Ca}^{2+}$  oscillation. When the spontaneous

contraction was terminated by treatment with BDM, the peak-to-peak intervals of  $\text{Ca}^{2+}$  oscillation were significantly increased. Figure B shows changes in the fluctuation of peak-to-peak intervals of  $\text{Ca}^{2+}$  oscillation assessed with the coefficient of variation (CV) of the intervals. Treatment with BDM tended to increase the fluctuation. Figures C1-C3 indicate the peak-to-peak amplitude of intracellular  $\text{Ca}^{2+}$  oscillation in cardiac myocytes in three different cultures. Treatment of cultures with 7.5 mM BDM did not change the peak-to-peak amplitude of intracellular  $\text{Ca}^{2+}$  oscillation significantly (C1 and C2). In some cases, the treatment actually increased the amplitude (C3). \* : statistically different compared with the control,  $P < 0.05$ .

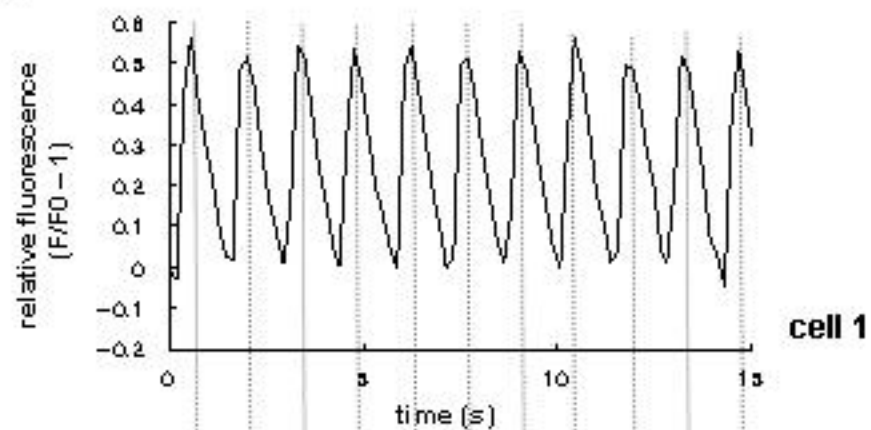
Fig. 5

Verapamil-induced termination of spontaneous cyclic contraction and intracellular  $\text{Ca}^{2+}$  oscillation in cultured cardiac myocytes. Treatment of cultures with 1  $\mu\text{M}$  verapamil, a blocker of L-type  $\text{Ca}^{2+}$  channels, for 5 min resulted in a reversible termination of the spontaneous cyclic contraction of the cardiac myocyte indicated by an arrow in the phase-contrast image of cell 1 in A1 (B2). Treatment with verapamil also terminated the intracellular  $\text{Ca}^{2+}$  oscillation in the same cell reversibly (C2). Both the spontaneous contraction and  $\text{Ca}^{2+}$  oscillation were terminated in almost all the cardiac myocytes treated with

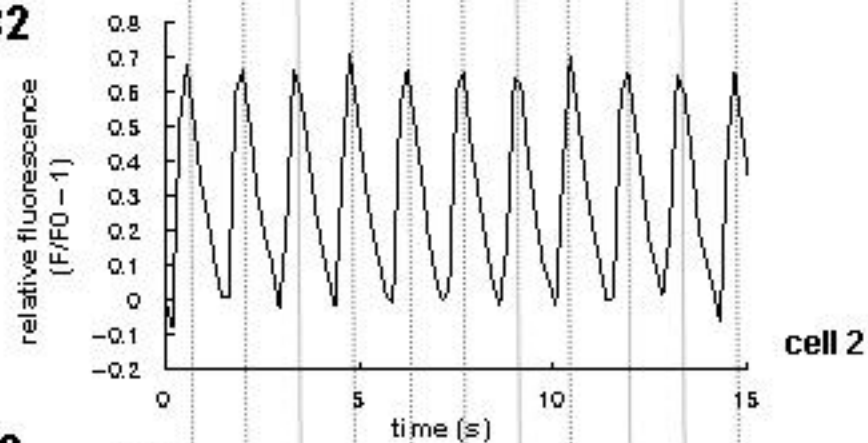
this concentration of verapamil ( $1 \mu\text{M}$ ) for 5 min. After the washing out of verapamil, both the spontaneous cyclic contraction (B3) and the  $\text{Ca}^{2+}$  oscillation (C3) gradually recovered. The scale bar indicates  $100 \mu\text{m}$ , and applies to both A1 and A2.



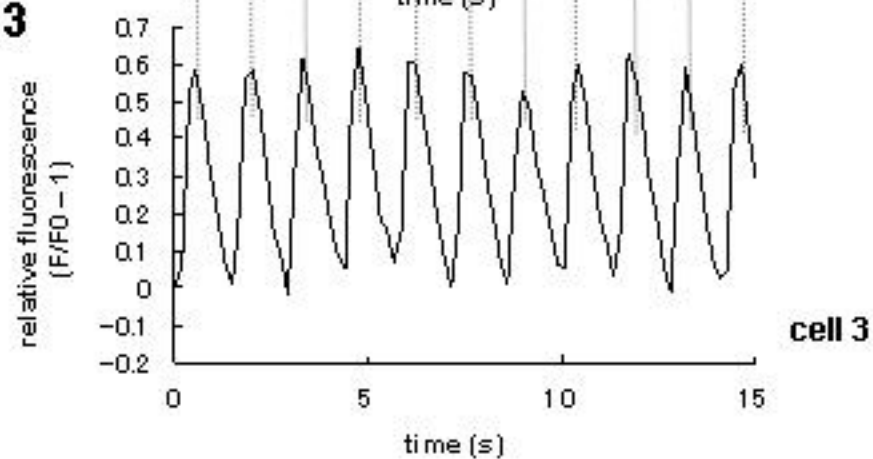
**B1**

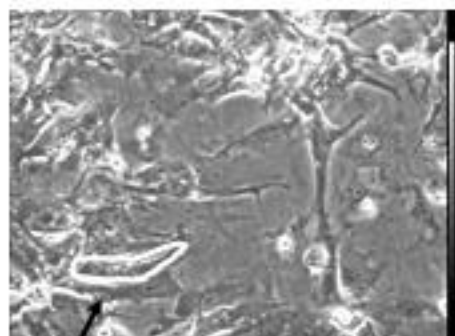
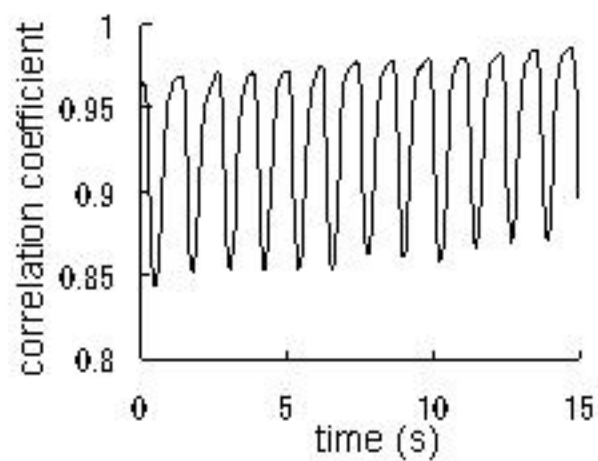
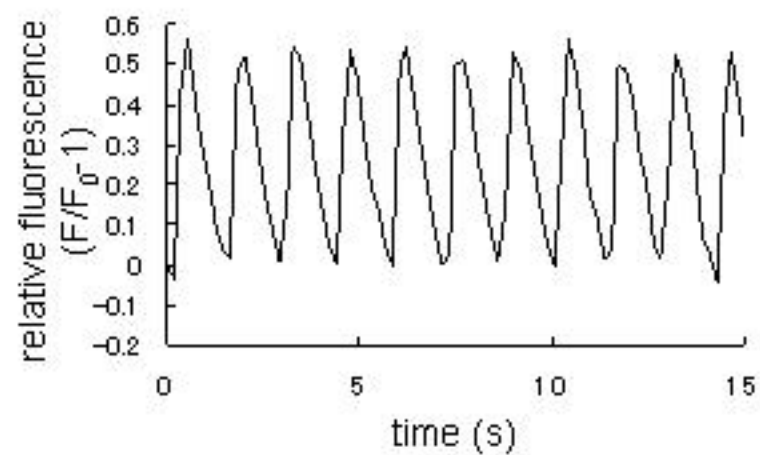
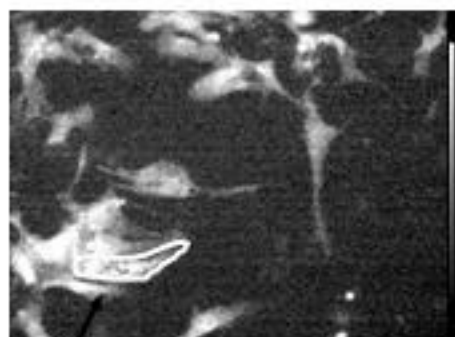
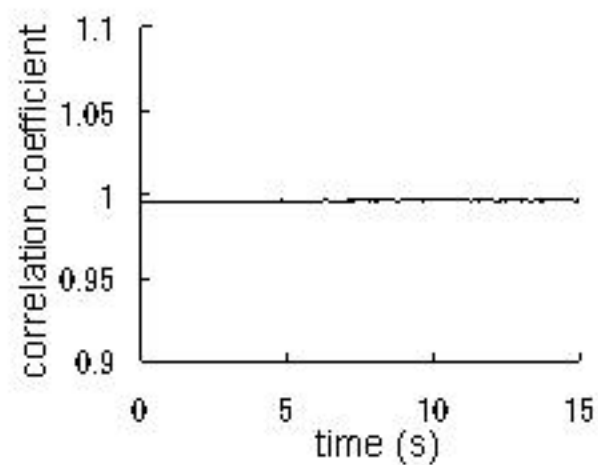
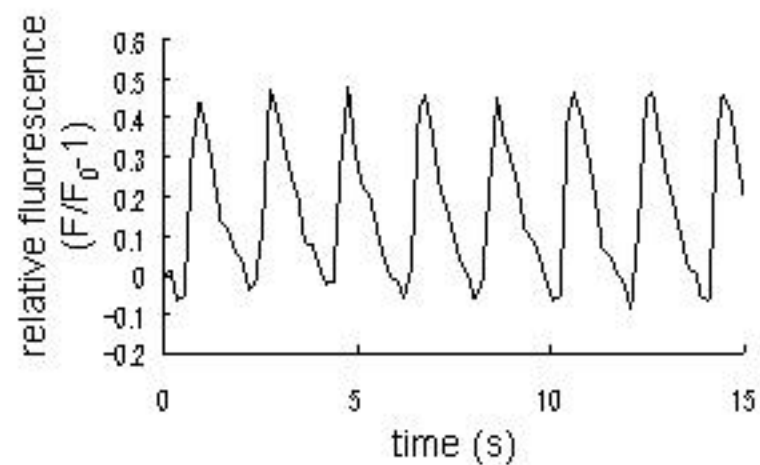


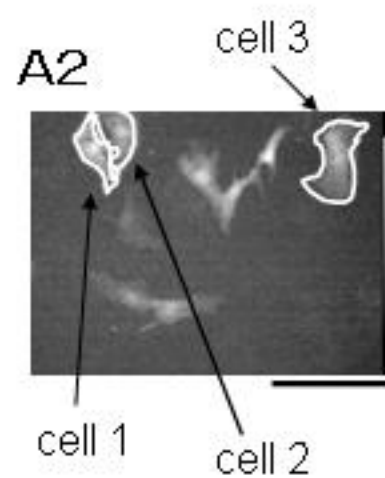
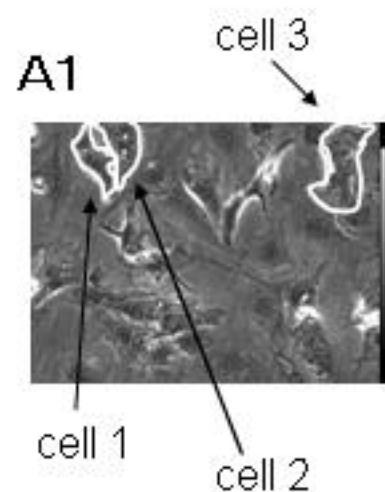
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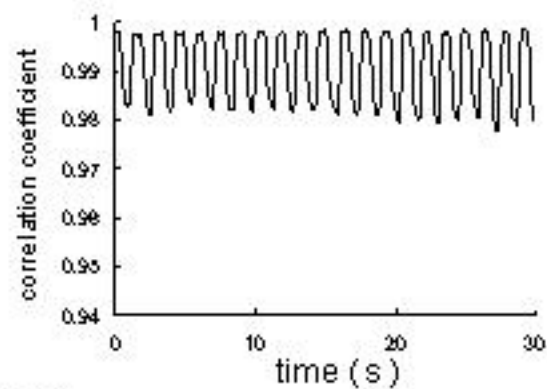
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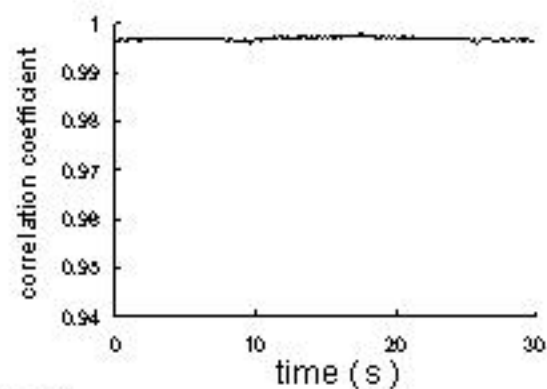
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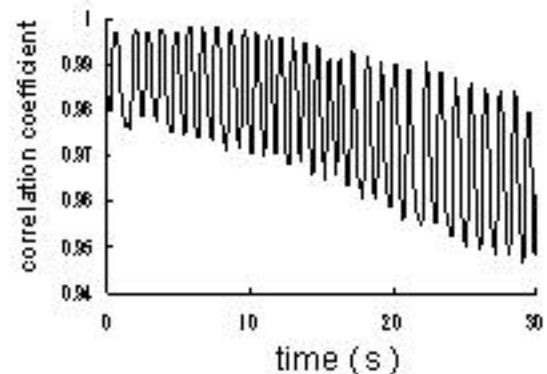
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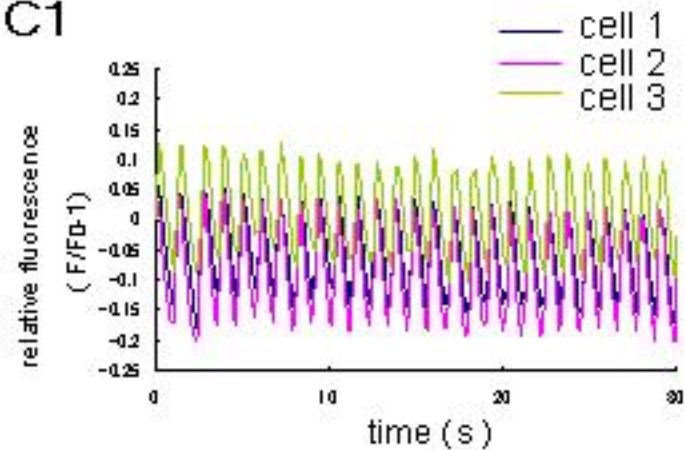
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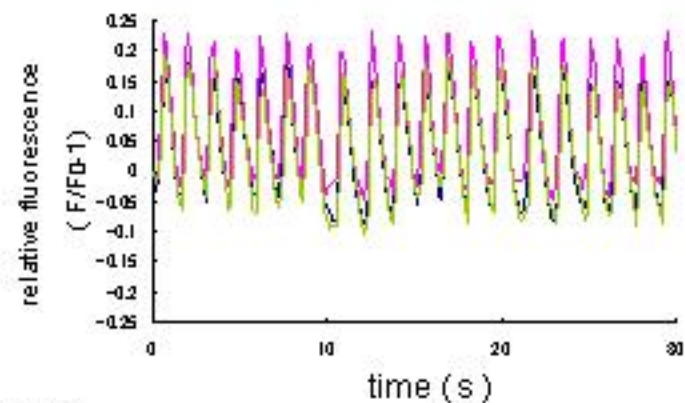
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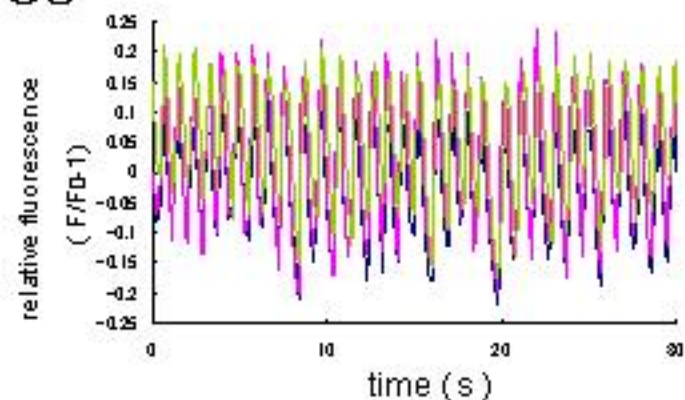
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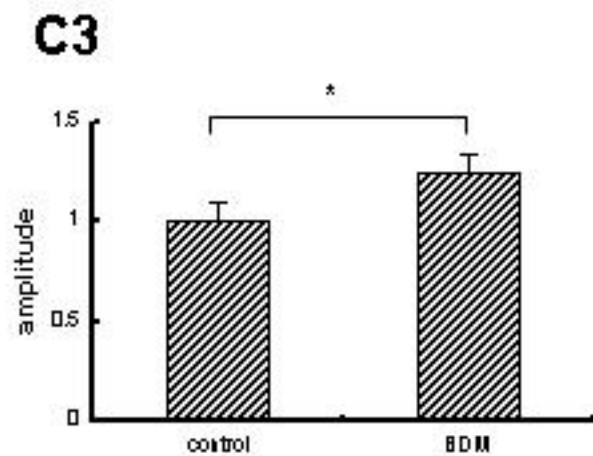
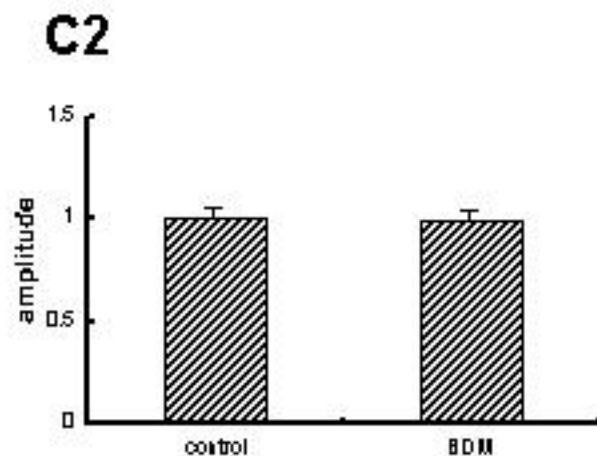
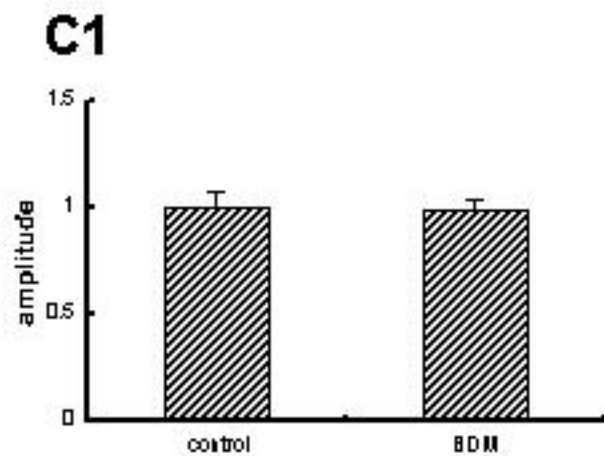
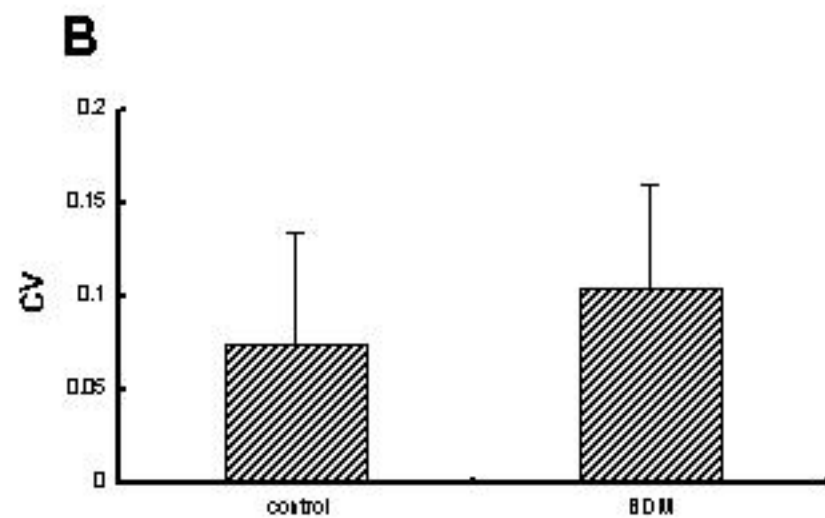
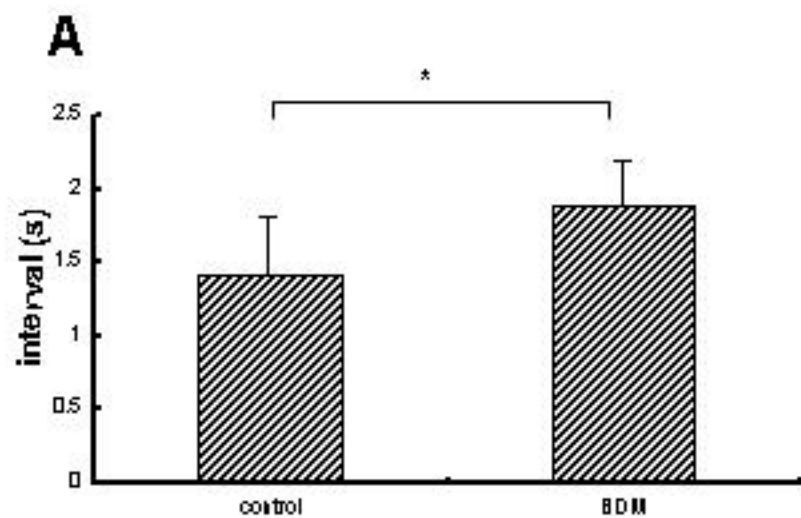


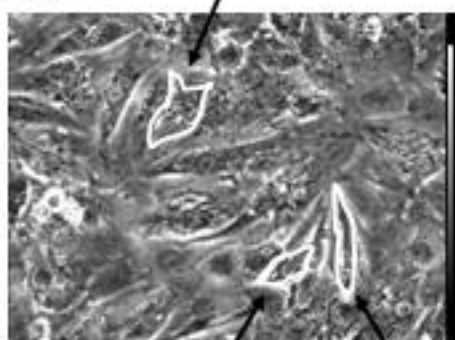
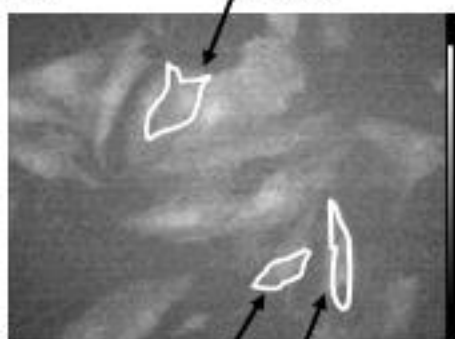
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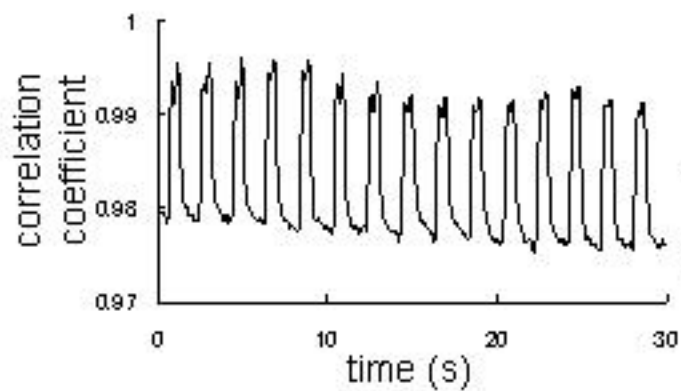
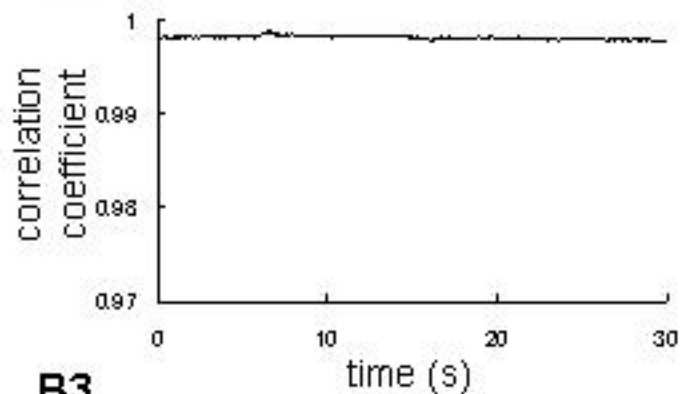
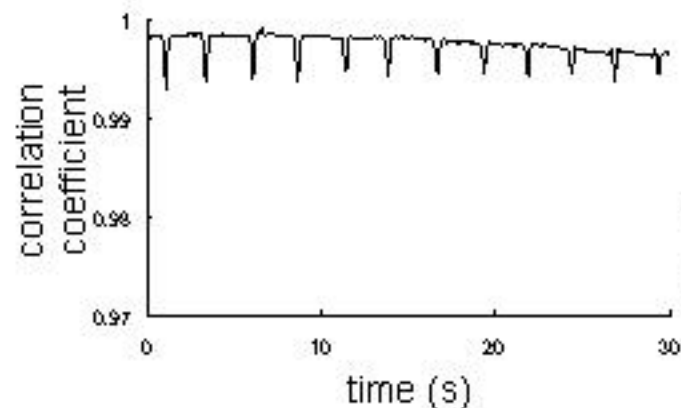
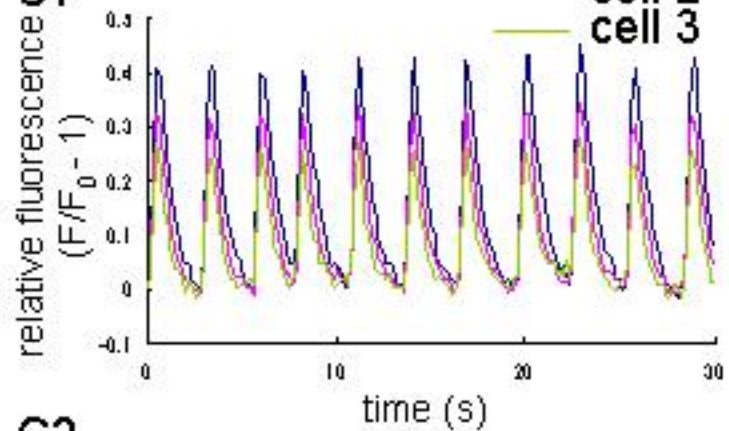
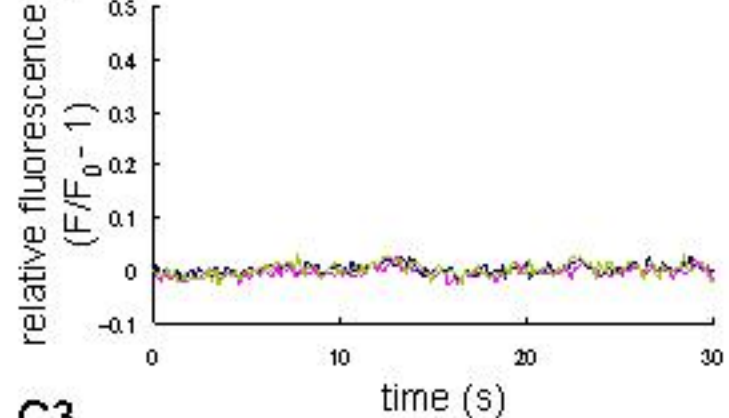
**C3**





**A1****A2**

cell 2  
cell 1

**B1****B2****B3****C1****C2****C3**