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Fast dual-excitation ratiometry with light-emitting diodes and high-speed liquid crystal shutters

Takashi Fukano, Satoshi Shimozono, Atsushi Miyawaki *

Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan

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Abstract

Dual-excitation ratiometric dyes permit quantitative measurements of Ca^{2+} concentrations ($[Ca^{2+}]$s), by minimizing the effects of several artifacts that are unrelated to changes in $[Ca^{2+}]$. These dyes are excited at two different wavelengths, and the resultant fluorescence intensities are measured sequentially. Therefore, it is difficult to follow fast $[Ca^{2+}]$ dynamics or $[Ca^{2+}]$ changes in highly motile cell samples. To overcome this problem, we have developed a new dual-excitation ratiometry system that employs two high-power light-emitting diodes (LEDs), two high-speed liquid crystal shutters, and a CCD camera. The open/close operation of the two shutters is synchronized with the on/off switching of the two LEDs. This system increases the rate at which ratio measurements are made to 1 kHz, and provides ratio images at 10–100 Hz depending on the signal intensity. We demonstrate the effectiveness of this system by monitoring changes in $[Ca^{2+}]$ in cardiac muscle cells loaded with Fura-2.

Keywords: Fluorescence imaging; Calcium imaging; Cardiac cell

Ratiometric imaging allows the quantitative measurement of intracellular ion concentrations [1]. Dual-emission ratiometry provides an ideal readout for fast confocal imaging without motion artifacts [2]. In contrast, dual-excitation ratiometry has one critical disadvantage. It is not possible to make simultaneous measurements that can be used for ratioing with this method. Therefore, sequential excitation at two different wavelengths is required to create a ratio image. This creates a time lag, which consists of the exposure time for the first image and the transition time required to alter the excitation wavelength. The latter is more than 50 ms for a mechanical filter wheel or about 1 ms for a monochromator-based wavelength-exchange system composed of a rotating diffraction grating attached to a galvanometer scanner. Although dual-excitation ratiometric Ca^{2+} dyes, such as Fura-2, BTC, Fura Red, and ratiometric-pericam [3], are widely used, it has been difficult to employ them in studies that monitor very fast Ca^{2+} dynamics or $[Ca^{2+}]$ changes in highly motile cells.

To obtain confocal images using ratiometric-pericam, we previously used a system in which two laser beams under the control of two acousto-optic tunable filters were alternated on every scanning line [4]. This system increased the rate at which ratio measurements could be made to 200 Hz and provided confocal images at 1–10 Hz depending on the image size.

To obtain ratiometric images without any time lag, we proposed a dual-excitation ratiometry method that used linearly polarized excitation light and polarization detection [5]. This method, however, is based on statistical features of the fluorescence polarization and is limited to samples that contain a large number of fluorophores. In addition, this method requires complicated calculations.

In the present study, we sought to develop an efficient, nearly simultaneous dual-excitation ratiometry system that allows us to rapidly switch between two synchronized

* Corresponding author. Fax: +81 48 467 5924.
E-mail address: matsushi@brain.riken.jp (A. Miyawaki).

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excitation-detection components by employing two high-power light-emitting diodes (LEDs) and two high-speed liquid crystal shutters.

Underlying principles

Fig. 1 illustrates a fluorescence microscopy system composed of two light sources (LS1 and LS2), two shutters (S1 and S2), and two CCD cameras (CCD1 and CCD2). The wavelengths of the light emitted by LS1 and LS2 correspond to the absorption maxima of a dual-excitable indicator. The light sources and shutters are electronically controlled in accordance with the following criteria.

(1) LS1 and LS2 are switched on and off at the same high frequency (around 1 kHz) but in anti-phase to each other.
(2) The opening and closing of S1 are synchronized with the switching on and off of LS1, respectively.
(3) The opening and closing of S2 are synchronized with the switching on and off of LS2, respectively.

With this configuration, the fluorescence images produced by the light emitted from LS1 and LS2 are obtained separately. When LS1 is on, the fluorescence emitted from the sample only reaches CCD1. Likewise, when LS2 is on, only CCD2 works as a detector. By repeating this operation during each CCD exposure period, fluorescence signals originating from the dual-excitable indicator excited by the light from LS1 and LS2 are delivered separately to CCD1 and CCD2, respectively, where they are integrated on a typical time scale (10–100 Hz).

Our new system features LEDs and liquid crystal shutters. A LED is a very attractive light source because of its low cost, compact size, and durability. Recently, high-power LEDs (more than 100 mW) and LEDs that emit various wavelengths of light including wavelengths in the ultraviolet region have become commercially available. In addition, the intensity of light from LEDs can be easily modulated at frequencies up to 100 MHz by current modulation. On the other hand, liquid crystal shutters are often used in sophisticated imaging experiments because they do not generate vibrations. Remarkably, a ferroelectric liquid crystal (FLC) [6] shutter can be used to produce exposure periods of as short as 200 μs, whereas a standard liquid crystal shutter offers an exposure time of about 10 ms. We were able to perform the aforementioned fast dual-excitation ratiometric imaging modality by synchronizing two sets of LEDs and FLC shutters.

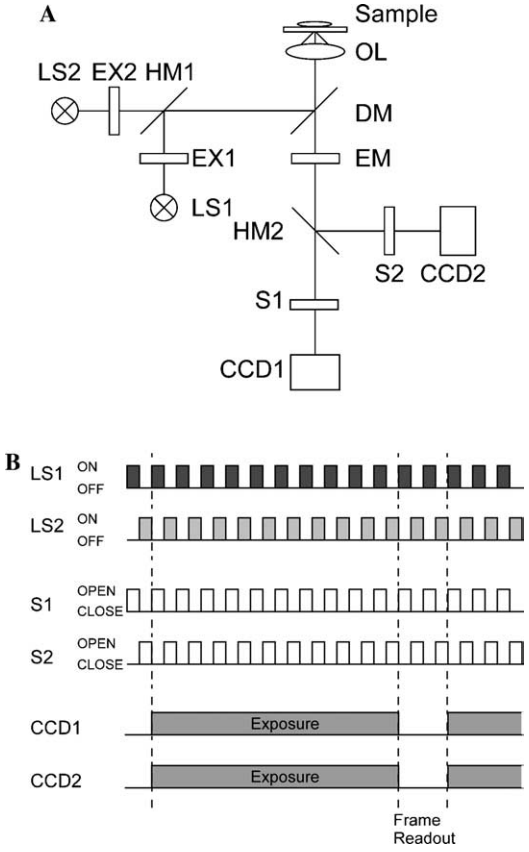

Fig. 1. An overview of fast dual-excitation ratiometry with synchronized excitation and detection. (A) A schematic diagram of the optical configuration. LS, light source; EX, excitation filter; HM, half mirror; DM, dichroic mirror; OL, objective lens; EM, emission filter; S, shutter. (B) A timing chart showing the on/off state of LS1 and LS2, the open/close state of S1 and S2, and CCD exposure and readout periods.

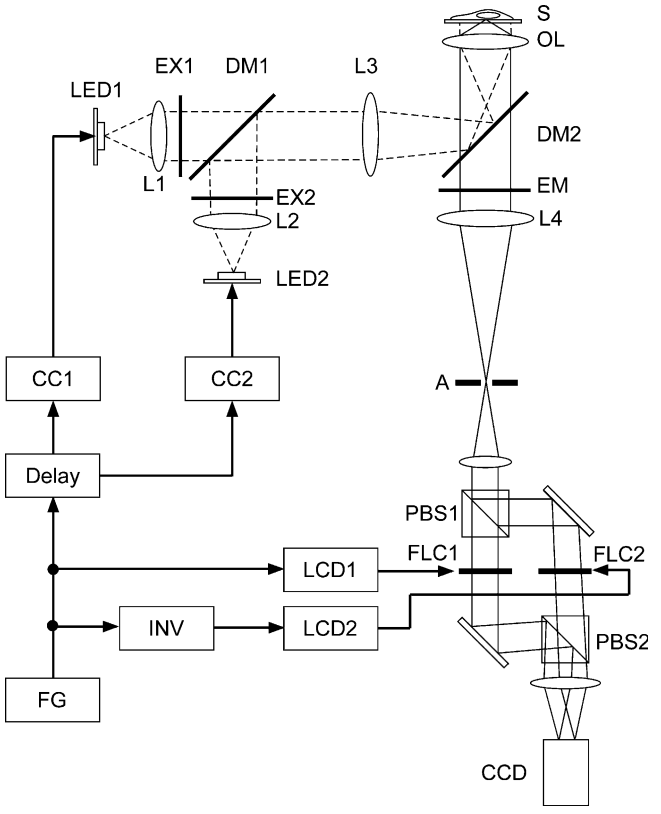

Fig. 2. A schematic of the optical and electronic configuration. LED, light-emitting diode; L, lens; EX, excitation filter; DM, dichroic mirror; OL, objective lens; S, sample; EM, emission filter; A, aperture; PBS, polarizing beam splitter; FLC, ferroelectric liquid crystal shutter; FG, function generator; CC, current controller; LCD, liquid crystal shutter driver; INV, inverter.

Materials and methods

Cell preparation. Cardiac muscle cells were prepared as described elsewhere [7]. The rat's heart was removed and rapidly placed in ice-cold phosphate-buffered saline containing 116 mM NaCl, 0.8 mM NaH_2PO_4, 5.3 mM KCl, 0.4 mM $MgSO_4$, 5 mM glucose, and 20 mM Hepes at pH 7.35. The hearts were subjected to four digestions with 0.125% trypsin. The first supernatant, which contained cell debris and blood cells, was discarded. Cell suspensions collected after each of the three subsequent digestions (20 min each) were pooled and resuspended in DMEM containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% FBS. Cells were then incubated for 1 h at 37 °C, allowing the selective attachment of non-myocytes. The unattached cells were plated at a density of 6×10^5 cells/ml onto 35-mm glass-bottomed dishes. On the next day, cells were washed and maintained in DMEM with 10% FBS. To load Fura-2 or Fura red into the cells, the acetoxymethyl ester form of the dye (Molecular Probes, Eugene, OR) was used. Dye loading was performed according to the manufacturer's instructions. Cardiac muscle cells were incubated at 37 °C for 30 min in a final working concentration of 2 or 10 μM for Fura-2. After the dye loading, the cells were washed twice with Hanks' balanced salt solution buffer and suspended in the same media. After 10 min, the cells were imaged while their temperatures were maintained at 37 °C with a heater (MI-IBC, Olympus, Shinjuku, Tokyo). For ratiometric-pericam, two or three days after cDNA transfection with FuGENE 6 (Roche Diagnostics, Grenzacherstrasse, Basal), cells in Hanks' balanced salt solution buffer were imaged at 37 °C.

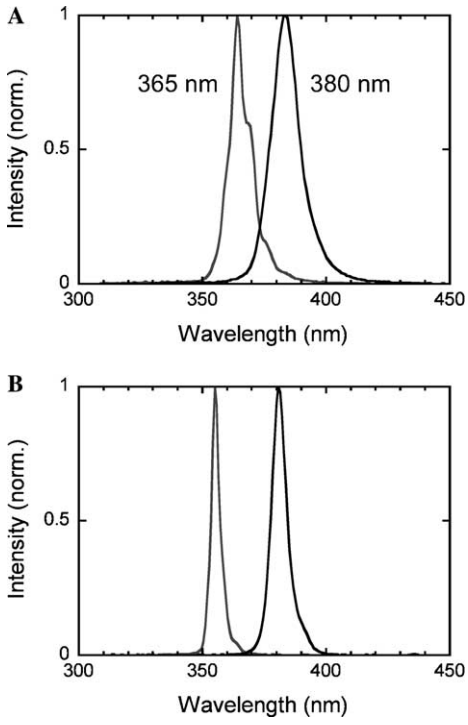

Fig. 3. (A) Spectral outputs of 365-nm band and 380-nm band LEDs. (B) The corresponding spectra filtered through 355- and 380-nm excitation filters.

LED emission measurements. LED emission spectra were measured with an optical spectrum analyzer (PMA-11, Hamamatsu Photonics, Hamamatsu, Shizuoka).

Experimental setup. Fig. 2 shows a diagram of the optical and electronic setup. An inverted fluorescence microscope (IX70, Olympus) was equipped with two high-power (~100 mW) ultraviolet LEDs. One was a 380-nm band LED (LED1; NCCU001, Nichia, Anan-Shi, Tokushima) that had a center wavelength of 383 and a 23-nm spectral bandwidth (the full width of the emitted spectra at the half-maximum intensity). The other was a 365-nm band LED (LED2; NCCU033, Nichia) that had a center wavelength of 364 and a 10-nm spectral bandwidth. Light emitted from LED1 and LED2 was collimated by lenses (L1 and L2, respectively) and filtered by two excitation filters: EX1 (380HT15, Omega Optical, Brattleboro, VT) and EX2 (CWL355, OptoSigma, Santa Ana, CA), respectively. To promote the excitation of Fura-2 by shorter wavelengths than its isosbestic wavelength (362 nm), EX2 was tilted 15° relative to the light pathway, which slightly shifted the transmission peak to a shorter wavelength. The normalized emitted spectra of LED1 and LED2 before and after filtration are shown in Fig. 3. The filtered light beams were combined by a dichroic mirror (DM1; 505DRLP, Omega Optical, 40% transmittance at around 380 nm) and reflected by a second dichroic mirror (DM2; 505DRLP-XR, Omega Optical). The light was applied to the samples through an objective lens (OL; UAPO/340, 40X, NA 1.35, Olympus). Köhler illumination allowed us to achieve uniform illumination. The fluorescence emitted from the samples passed through the OL, DM2, an emission filter (EM; 480ALP, Omega Optical), and a tube lens (L4) before it was focused onto a 4.6 mm × 3.1 mm rectangular aperture (A). After being trimmed by the aperture, the light beam was split with a polarizing beam splitter (PBS1). FLC shutters (FLC1 and FLC2; LV2500P-OEM, DisplayTech, Longmont, CO) were inserted in the two optical paths. The azimuth angles of the FLC shutters were adjusted so that the maximum transmittance was obtained in the open state. Then the two light beams were recombined with a small offset and projected onto a single CCD chip of a cooled camera (Cascade 512B, Roper Scientific, Tucson, AZ). Image acquisition and subsequent image processing were performed using imaging software (MetaMorph 6, Universal Imaging, Media, PA). We used the "stream acquisition mode" of MetaMorph, by which images are captured as rapidly as possible in a continuous data stream and stored in the random access memory of a computer. The rate of image acquisition was 31 ms/frame when the binning on the camera was set to 4. A function generator (FG; AFG320, Sony Tektronix, Shinagawa, Tokyo) provided synchronized signals for the whole system. LED and FLC switching were all synchronized at 1 kHz with the reference square wave.

LED1 and LED2 were driven using home-built current controllers (CC1 and CC2). The output power of the LEDs was tuned by con-

Table 1
A list of LEDs, excitation filters, dichroic mirrors, and emission filters for Fura-2, Fura red, and ratiometric-pericam

Indicator	LED1	EX1	LED2	EX2	DM1	DM2	EM
Fura 2	NCCU033[a] (380 nm)	380HT15	NCCU001[a] (365 nm)	CWL355[d]	505DRLP	505DRLPXR	480ALP
Fura red	LXHL-NE98[b] (505 nm)	BP480-500[c]	TS[a] (400 nm)	405DF40	435DRLP	505DRLPXR	530ALP
Ratiometric-pericam	LXHL-NE98[b] (505 nm)	BP480-500[c]	TS[a] (400 nm)	405DF40	435DRLP	505DRLPXR	530ALP

Otherwise: Omega Optical; TS, technical sample.
[a] Nichia.
[b] Lumileds.
[c] Olympus.
[d] OptoSigma.

trolling the injection currents. The reference signal from the FG was fed into a delay circuit (DG535, Stanford Research Systems, Sunnyvale, CA), which generated controlling signals for LED1 and LED2. To operate the two FLCs, the signal from the FG was divided into two. One signal was directly delivered to the first FLC driver (LCD1; DR95, Displaytech, Longmont, CO), whereas the other signal was fed into the second FLC driver (LCD2) through an inverter circuit (INV). Thus, the open/close states of the two FLC shutters were also switched in anti-phase to each other. This setup was used for Fura-2. For other dual-excitable indicators, appropriate LEDs and filters are substituted into the system. The LEDs, excitation filters, dichroic mirrors, and emission filters for Fura Red and ratiometric-pericam are summarized in Table 1.

Results and discussion

Spontaneously beating cultured cardiac muscle cells were loaded with Fura-2 and imaged with our new system. Due to the motility of these cells and the relatively fast changes in $[Ca^{2+}]$, the dynamic aspects of Ca^{2+} regulation could not be analyzed by standard dual-excitation ratiometry. Fig. 4A shows fluorescence signals from samples excited at 355 nm (left side) and 380 nm (right side). Temporal profiles of the averaged fluorescence intensities obtained in the region inside the squares in Fig. 4A are shown in Fig. 4B. When LED1 (380-nm excitation) was switched off (indicated by the arrow a), the 380-nm signal dropped to zero, whereas no change in the 355-nm signal was detected. Conversely, switching LED2 off affected only the 355-nm signal (indicated by the arrow b). This confirmed complete separation of the 355- and 380-nm signals. Fig. 4C shows fluorescence intensity profiles obtained in the same region after 60 s. The reciprocal change between the 355- and the 380-nm signals was evident during a contraction/relaxation cycle. The ratio of the 355-nm signal to the 380-nm signal in Fig. 4C was plotted (Fig. 4D). The intensity change of the 355-nm signal was relatively small compared with that of the 380-nm signal, probably because 355 nm was still close to the isosbestic wavelength of Fura-2. Thus, a 340-nm band UV-LED [8,9] should be used for the shorter wavelength peak of Fura-2.

Next, we obtained two-dimensional images at video-rate of cytosolic $[Ca^{2+}]$ in cardiac cells (Fig. 5). A fluorescence image of a cell is given in Fig. 5A. Fig. 5B shows a series of subsequent ratio images. A local and transient increase in $[Ca^{2+}]$ (indicated by an arrowhead) was observed from 62 to 217 ms and from 5270 to 5456 ms, neither of which caused the cell to contract. A global increase in $[Ca^{2+}]$ suddenly appeared at 775 ms and lasted until 2170 ms. During this period, a significant contracting movement of the cell was observed. The abrupt increase in $[Ca^{2+}]$ suggests a positive feedback regulation of Ca^{2+} mobilization, which may be required for efficient contraction. The contraction-coupled $[Ca^{2+}]$ increase was followed by a local $[Ca^{2+}]$ increase, which faded away. A similar robust increase in $[Ca^{2+}]$ was observed from 3193 to 4278 ms.

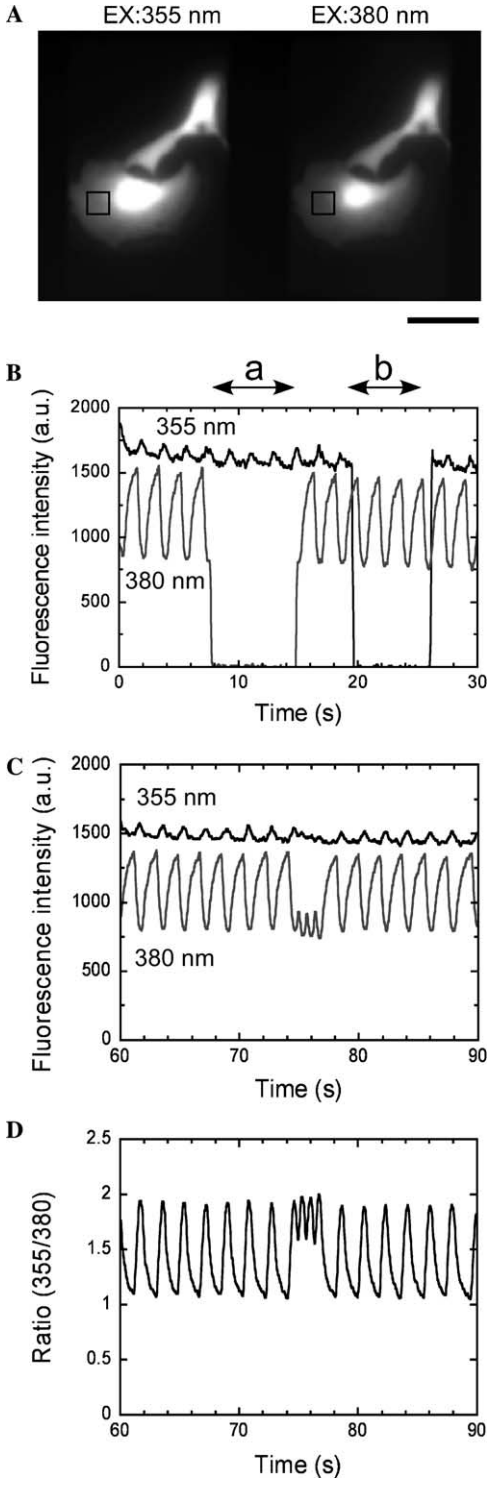

Fig. 4. Ratiometric measurement of $[Ca^{2+}]$ by the fast dual-excitation ratiometry. (A) Fluorescence images of a spontaneously beating cultured cardiac muscle cell loaded with Fura-2 and excited at 355 nm (left side) and 380 nm (right side). Scale bar, 20 μm. (B) Fluorescence intensities obtained by excitation at 355 nm (dark trace) and 380 nm (light trace) in a region indicated by the squares in (A). LED1 (380 nm) and LED2 (365 nm) were switched off during the time indicated by the arrows (a) and (b), respectively. (C) Fluorescence signals for 355- and 380-nm excitation in the same regions after 60 s. (D) Ratio of the 355-nm signals to the 380-nm signals.

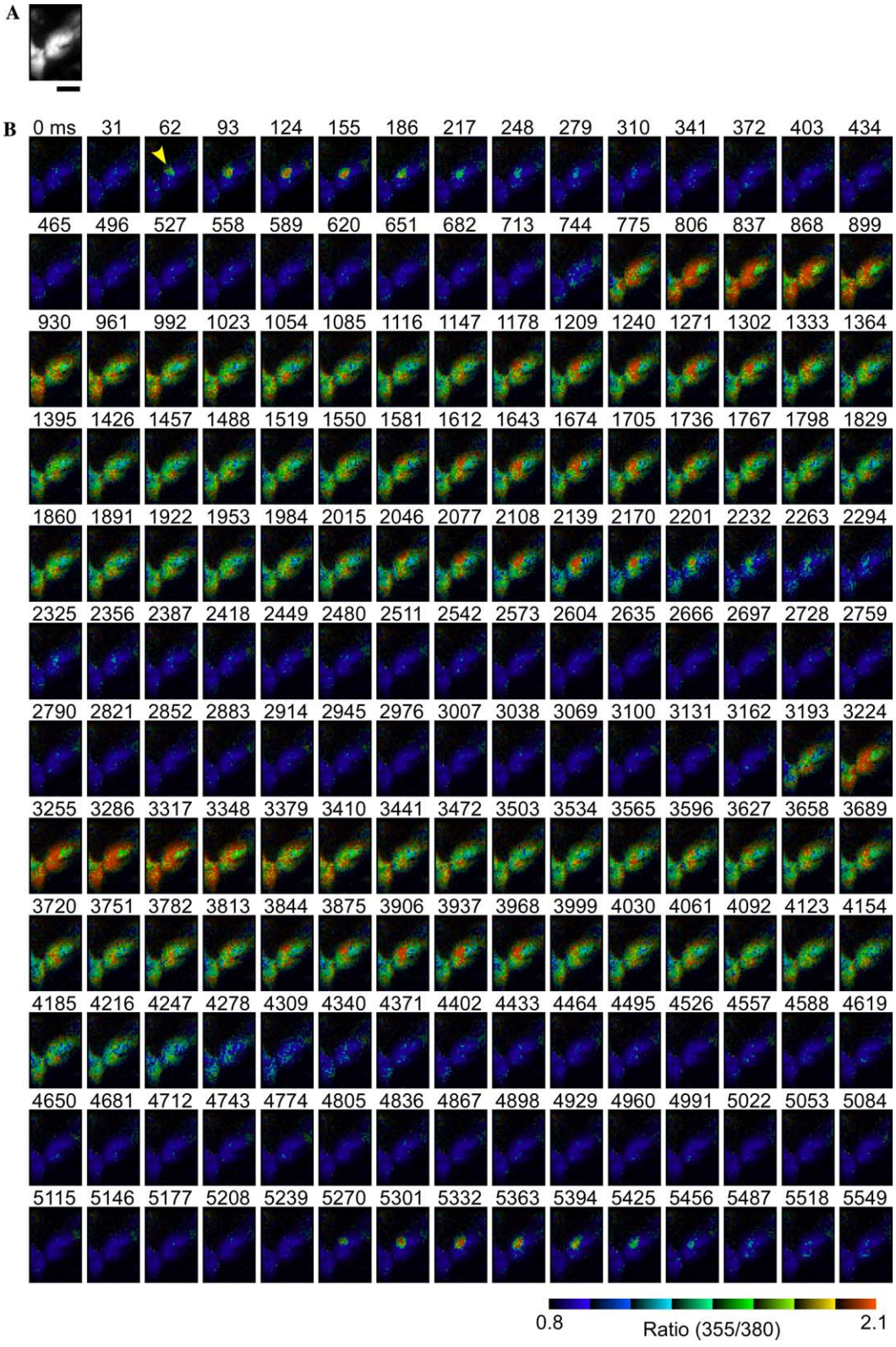

Fig. 5. (A) A fluorescence image obtained by excitation at 355 nm. Scale bar, 20 μm. (B) Ratio images obtained every 31 ms of Ca^{2+} movements in spontaneously beating cardiac cells. The time above each image indicates the elapsed time.

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