Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59881R4
Full Title:	A novel nicotinamide adenine dinucleotide correction method for intracellular Ca2+ measurement with fura-2-analog in live cells
Keywords:	Mitochondria; Calcium; Fura-2-FF; Mitochondrial membrane potential; NADH; pH; Calibration equation
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Songpa-gu,Seoul,Korea

TITLE:

A Novel Nicotinamide Adenine Dinucleotide Correction Method for Intracellular Ca^{2+} Measurement with Fura-2-Analog in Live Cells

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KEYWORDS:

mitochondria, calcium, fura-2-FF, mitochondrial membrane potential, NADH, pH, calibration equation

SUMMARY:

Due to the spectral overlapping of the excitation and emission wavelengths of NADH and fura-2 analogs, the signal interference from both chemicals in live cells is unavoidable during quantitative measurement of [Ca^{2+}]. Thus, a novel online correction method of NADH signal interference to measure [Ca^{2+}] was developed.

ABSTRACT:

To measure [Ca^{2+}] quantitatively, fura-2 analogs, which are ratiometric fluoroprobes, are frequently used. However, dye usage is intrinsically limited in live cells because of autofluorescence interference, mainly from nicotinamide adenine dinucleotide (NADH). More specifically, this is a major obstacle when measuring the mitochondrial [Ca^{2+}] quantitatively using fura-2 analogs because the majority of NADH is in the mitochondria. If the fluorescent dye concentration is the same, a certain excitation intensity should produce the same emission intensity. Therefore, the emission intensity ratio of two different excitation wavelengths should be constant. Based on this principle, a novel online correction method of NADH signal interference to measure [Ca^{2+}] was developed, and the real signal intensity of NADH and fura-2 can be obtained. Further, a novel equation to calculate [Ca^{2+}] was developed with isosbestic excitation or excitation at 400 nm. With this method, changes in mitochondrial [Ca^{2+}] could be successfully measured. In addition, with a different set of the excitation and emission wavelengths, multiple parameters, including NADH, [Ca^{2+}], and pH or mitochondrial membrane

potential (Ψ_m), could be simultaneously measured. Mitochondrial [Ca^{2+}] and Ψ_m or pH were measured using fura-2-FF and tetramethylrhodamine ethyl ester (TMRE) or carboxy-seminaphtorhodafluor-1 (carboxy-SNARF-1).

INTRODUCTION:

The significant role of intracellular Ca^{2+} is widely known[1]. The quantification of [Ca^{2+}] is essential to understand the processes of the cellular physiological functions. Fura-2 analogs are quite useful because they are excited in the UV range (<400 nm), and the ratiometric method can be applied for the quantitative measurement. Therefore, other physiological parameters such as pH, membrane potential, etc., can be measured with other fluorescent dyes. The mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) range was reportedly 0.08−20 μM[2-5]. Among fura-2 analogs, fura-2-FF is appropriate for measuring this range of [Ca^{2+}]. However, the live cells unfortunately contain NADH/NADPH for their metabolic processes, and NADH generates signal interference because of the overlapping excitation and emission spectra with the fura-2 analog. This interference greatly limits the use of fura-2 analogs. Specifically, if the analog is applied to measure mitochondrial [Ca^{2+}], this interference is the biggest obstacle because the highest amount of NADH is in the mitochondria. This is further complicated by NADH changes being related to the mitochondrial membrane potential (Ψ_m) and the change of Ψ_m affects $[Ca^{2+}]_m$[6-9]. Furthermore, for studying $[Ca^{2+}]_m$ dynamics, it is essential to know the status of other mitochondrial parameters, such as NADH, Ψ_m, and pH.

The emissions at 450 nm and 500 nm with excitations at 353 nm, 361 nm, and 400 nm contain the signals from NADH and fura-2-FF, and the equations are as follows. Herein, 353 nm and 361 nm are the isosbestic points of fura-2-FF for emissions at 450 nm and at 500 nm, respectively.

$$F_{361,450} = F_{361,450,NADH} + F_{361,450,Fura} \quad \text{Equation 1}$$

$$F_{353,500} = F_{353,500,NADH} + F_{353,500,Fura} \quad \text{Equation 2}$$

$$F_{400,500} = F_{400,500,NADH} + F_{400,500,Fura} \quad \text{Equation 3}$$

where $F_{x,y}$ is the measured emission intensity at y-nm by x-nm excitation, $F_{x,y,NADH}$ represents the pure NADH-dependent emission intensity, and $F_{x,y,Fura}$ represents the pure fura-2-FF-dependent emission intensity. Under the same concentration of the fluorescent dye, a certain excitation intensity should produce the same emission intensity. Therefore, the emission intensity ratio of two different excitation wavelengths should be constant. Ca^{2+} and fura-2 did not affect NADH fluorescence characteristics; therefore, the ratio of the emission at 450 nm and at 500 nm of NADH was constant at any excitation wavelength. The same rule can be used for fura-2-FF based on the assumption that NADH or [Ca^{2+}] does not affect the emission and excitation spectra of fura-2-FF. However, Ca^{2+} caused a spectral shift of the fura-2-FF emission. Therefore, to remove the effect of Ca^{2+}, isosbestic excitation, which is independent of Ca^{2+}, needs to be used. Each emission wavelength (i.e., 450 nm and 500 nm) has a different isosbestic point, and from our experimental setup, 353 nm at 500 nm and 361 nm at 450 nm were chosen. From these, the following equations are valid[10].

$$R_f = F_{361,450,Fura}/F_{353,500,Fura} \quad \text{Equation 4}$$

$R_{N1} = F_{400,500,NADH}/F_{361,450,NADH}$ Equation 5

$R_{N2} = F_{353,500,NADH}/F_{361,450,NADH}$ Equation 6

With these constants, the following equations from (Equation 1) (Equation 2), and (Equation 3) are valid.

$F_{361,450} = F_{361,450,NADH} + R_f \times F_{353,500,Fura}$ Equation 7

$F_{353,450} = R_{N2} \times F_{361,450,NADH} + F_{353,500,Fura}$ Equation 8

$F_{400,500} = R_{N1} \times F_{361,450,NADH} + F_{400,500,Fura}$ Equation 9

From these equations, if R_f, R_{N1}, and R_{N2} are known, pure signals of NADH and fura-2 can be obtained as follows.

$F_{361,450,NADH} = (F_{361,450} - R_f \times F_{353,500})/(1 - R_f \times R_{N2})$ Equation 10

$F_{353,500,Fura} = (R_{N2} \times F_{361,450} - F_{353,500})/(R_f \times R_{N2} - 1)$ Equation 11

$F_{400,500,Fura} = F_{400,500} - R_{N1} \times F_{361,450,NADH}$ Equation 12

$R_{Fura} = F_{353,500,Fura}/F_{400,500,Fura}$ Equation 13

The Ca^{2+}-bound form of fura-2-FF was practically non-fluorescent at the 400 nm excitation wavelength. Based on this property, the following new calibration equation can be derived.

$[Ca^{2+}] = K_d \cdot (F_{400,500,max}/F_{353,500,max}) \times (R_{Fura} - R_{min})$ Equation 14

where K_d is a dissociation constant, $F_{400,500,max}$ and $F_{353,500,max}$ are the maximum values of the emitted signals at 500 nm with excitations at 400 nm and 353 nm, respectively, and R_{min} is the minimum R_{Fura} in Ca^{2+}-free condition. Since the isosbestic excitations were used, the equation can be simplified further as follows.

$[Ca^{2+}] = K_d \cdot (1 / R_{min}) \cdot (R_{Fura} - R_{min})$ Equation 15

Therefore, only K_d and R_{min} values are required to calculate $[Ca^{2+}]$.

PROTOCOL:

All experimental protocols were approved by the local institutional animal care and use committee.

1. Solution preparation

1.1. Prepare single freshly isolated cardiac myocytes[11].

NOTE: Each laboratory might have a different cell storage solution. Here, the myocytes are stored in culture medium (DMEM).

1.2. Prepare 100 mL of Ca^{2+}-free solution (**Table 1**).

1.3. Prepare 50 mL of culture medium in a 50 mL beaker. Aliquot 5 mL and put it in a water bath at 37 °C. Keep the remaining solution at room temperature.

1.4. Prepare 50 mL of the saponin solution by adding 5 mg of saponin to 50 mL of Ca^{2+}-free solution.

NOTE: Saponin is used to permeabilize cardiac myocytes, to remove cytosolic compartments, and to visualize the mitochondrial fluorescence only.

1.5. Prepare 16 µL of 1 mM fura-2-FF-AM dissolved in dimethyl sulfoxide (DMSO).

NOTE: Make 1 mM stock solution of fura-2-FF-AM dissolved in DMSO and aliquot 16 µL in a 2 mL tube. Store them at -20 °C until use.

1.6. Prepare 50 mL of NADH-free Ca^{2+}-free solution (**Table 1**) and 50 mL of NADH-free Ca^{2+}-saturated solution (**Table 1**) when isosbestic points are to be measured. Adjust pH to 7.0 with KOH.

NOTE: NADH-free Ca^{2+}-free solution (**Table 1**) contains 10 µM FCCP and 100 µM ADP without any mitochondrial substrates to minimize NADH in mitochondria.

1.7. Prepare 50 mL of Ca^{2+}-free solution, 50 mL of malate solution, 50 mL of pyruvate solution, 50 mL of malate-pyruvate solution, and 50 mL of rotenone solution to be used for NADH correction factor measurements (**Table 1**).

2. Fluoroprobe loading procedure into the mitochondria

2.1. Prepare the dye-loading solution by adding 2 mL of the culture medium to 16 µL of 1 mM fura-2-FF-AM.

NOTE: Fluorescent dye is fragile under the light. Prepare the solution just before use. Keep the solution containing the fluorescent dye in a dark place. The final concentration of fura-2-FF-AM is 8 µM. If carboxy-SNARF-1 was used, prepare the dye loading solution with 2 µM carboxy-SNARF-1-AM.

2.2. Take 2 mL of the isolated cells and place in a 5 mL test tube in an upright position.

2.3. Wait 15 min for myocytes to sink to the bottom and remove the supernatant.

NOTE: The supernatant may contain cell debris. Do not centrifuge the tube to avoid cell damage.

2.4. Add 2 mL of the dye-loading solution.

2.5. Incubate the dye-loading solution with cells for 60 min at 4 °C.

2.6. Then, put the test tube in a 37 °C water bath for 30 min in an upright position.

2.7. Remove the supernatant, and add 4 mL of the prewarmed culture medium of 37 °C. Incubate the cells for 60 min in a 37 °C water bath.

2.8. Finally, remove the supernatant, add 4 mL of culture medium at room temperature and keep the tube at room temperature.

3. Introduction of the multiparametric measurement system

NOTE: **Figure 1** shows a diagram of the whole system.

3.1. For an excitation light source, use a fast monochromator (polychrome II) that can change the light within 3 ms.

3.2. Use an oil immersion lens (40x, NA 1.3) with an inverted microscope to increase the signal intensity.

3.3. Use a near-infrared filter and a charge-coupled device (CCD) camera to monitor the object field without fluorescent signal interference.

3.4. Capture the object field image to get the area.

3.5. Adjust the object field in monitor screen with a field diaphragm just to show the cell for reducing the background.

3.6. Use four photomultiplier tubes with each band-pass filter (450, 500, 590, and 640 nm) to detect emission wavelengths with photon counting method. Use the appropriate dichroic mirrors to split and to redirect the emission light.

NOTE: The excitation light is very strong compared to the emission light. Thus, choose the band-pass filter with the highest blocking characteristics to reduce the background. A photon counting system comprises a combination of PMTs, photon counter units, and a high-speed counter. To control the system and to sample the data, a custom-made driving software was used. Finding a way to apply this method with other systems is necessary.

4. NADH correction methods with a multiparametric measurement system

4.1. The background signal detection and the correction methods with the cell area

NOTE: There are two kinds of backgrounds. One comes from the cells and the other comes from

the reflection on the cover slip (the cell-free background). Both backgrounds need to be corrected in each experiment.

4.1.1. Mount the dye-free cells in the bath on the microscope and wait 3 min for cells to sink to the bottom. Perfuse NADH-free Ca^{2+}-free solution for around 5 min.

NOTE: The perfusion rate of all solutions is 2-3 mL/min at 37 °C. Adjust cell numbers to see around one cell per one objective field with a 40x objective lens.

4.1.2. Set the object field to cover the targeted cell.

4.1.3. After moving the cell out of the field, measure the background signals of the cell-free window and set them as offsets.

NOTE: The signal means the light signal to be detected in the photon counting system.

4.1.4. Return the cell to the initial position and measure the cell background signals and the cell area.

NOTE: Even though the excitation light is filtered with the bandpass filter, it still contains a large amount of the filtered light. This light is dispersed when hitting the cells and causes considerable background signals because the photon counting system is highly sensitive. It needs to be corrected. The cell area may be calculated with a captured cell image and available imaging software. The unit of the cell area can be any unit including pixel count. Only standardization is necessary.

4.1.5. Repeat steps 4.1.1 to 4.1.4 10 times to obtain the relationship between the cell area and the cell background signals.

NOTE: Later, the cell background signals can be calculated from the cell area from the relationship. Since the excitation light bulb ages, this procedure needs to be repeated, at least, every month.

4.2. Identification of the isosbestic points of fura-2-FF in situ

NOTE: Many reports have stated that fluorescent characteristics are changed in cells. Therefore, perform all procedures to obtain the parameters to correct the interference in situ.

4.2.1. Mount the dye-loaded cell on the microscope and wait 3 min for cells to sink to the bottom.

NOTE: Adjust cell numbers to see around one cell per one objective field with a 40x objective lens.

4.2.2. Perfuse the NADH-free Ca^{2+}-free solution at 37 °C.

4.2.3. After targeting the cell, measure the cell-free background and the cell area as shown in section 4.1. Calculate the cell background from the cell area.

NOTE: Both background signals need to be corrected in each experiment.

4.2.4. Perfuse the saponin solution for 60 s and return to the NADH-free Ca^{2+}-free solution.

4.2.5. Measure fura-2-FF-emitted signals at 450 nm and 500 nm simultaneously by the excitation scan from 350 nm to 365 nm with a 0.1 nm step.

4.2.6. Perfuse the NADH-free Ca^{2+}-saturated solution and repeat step 4.2.5.

4.2.7. Subtract the signals in the Ca^{2+}-saturated solution from the signals in the Ca^{2+}-free conditions.

4.2.8. Repeat steps 4.2.2 to 4.2.7 with other single cardiac myocytes.

NOTE: If the signal intensity become weaker, repeat from step 4.2.1. Repeat the procedure for, at least, 5 different cells.

4.2.9. From all obtained signals, calculate the standard deviations of the emission at each excitation and choose the excitation wavelength, showing the minimum standard deviation (SD) value as an isosbestic point.

NOTE: The representative figures are shown in **Figure 2**.

4.3. Measurement of R factors

4.3.1. Calculate R_f with equation 4 from the signals obtained in section 4.2.

4.3.2. Mount the dye-free cells on the microscope and perfuse the Ca^{2+}-free solution.

4.3.3. Measure the signals such as $F_{361, 450, NADH}$, $F_{400, 500, NADH}$, $F_{361, 450, NADH}$, and $F_{353, 500, NADH}$.

4.3.4. Perfuse the malate solution. Repeat step 4.3.3 and measure the signals.

4.3.5. Perfuse the pyruvate solution. Repeat step 4.3.3 and measure the signals.

4.3.6. Perfuse the malate-pyruvate solution. Repeat step 4.3.3 and measure the signals.

4.3.7. Perfuse the rotenone solution. Repeat step 4.3.3 and measure the signals.

NOTE: The example of the NADH signal recorded on 5 mM pyruvate, 5 mM malate plus 5 mM pyruvate, and 10 µM rotenone addition is shown in **Figure 3**.

4.3.8. Calculate each slope of $F_{361, 450, NADH}$ vs. $F_{400, 500, NADH}$ and $F_{361, 450, NADH}$ vs. $F_{353, 500, NADH}$. As shown in **Figure 3**. Each slope indicates R_{N1} and R_{N2}.

5. Selection of the excitation and the emission light for TMRE or carboxy-SNARF-1

5.1. If TMRE for measuring the mitochondrial potential was used in addition, use the 530 nm excitation wavelength and the 590 nm emission wavelength.

5.2. If carboxy-SNARF-1 for measuring the mitochondrial potential was used in addition, use the excitation wavelength of 540 nm and emission wavelengths of 590 nm and 640 nm[12].

6. Selection of K_d value of fura-2-FF

6.1. The change of pH can affect K_d values for Ca^{2+} binding on fura-2-FF[10]. Use the K_d value of 5.28 at pH 7.5 for the mitochondria.

REPRESENTATIVE RESULTS:

Mitochondrial Ca^{2+} changes due to correction[10]

Figure 4 shows the changes in $[Ca^{2+}]_m$ before and after the correction. The results clearly showed the substantial changes in $[Ca^{2+}]_m$. The mitochondrial resting calcium concentration without cytosolic Ca^{2+} ($[Ca^{2+}]_c$) was 1.03 ± 0.13 µM (mean ± S.E., n = 32), and the maximum $[Ca^{2+}]_m$ at 1-µM $[Ca^{2+}]_c$ was 29.6 ± 1.61 µM (mean ± S.E., n = 33) (**Figure 5**).

Simultaneous measurement of NADH, $[Ca^{2+}]$, and Ψ_m[10]

A positively charged TMRE can be distributed in a membrane potential-dependent manner. Membrane potential can be calculated using the Nernst's equation with the concentration in each compartment. The mitochondrial TMRA was monitored with the perfusion of 2-nM TMRE. The initial Ψ_m was assumed to be −150 mV, and the change of Ψ_m was calculated based on that. The application of Ca^{2+} decreased NADH but affected Ψ_m only negligibly (**Figure 6**).

Mitochondrial pH changes by the change in $[Ca^{2+}]m$[10]

The mitochondrial pH with the additional loading of carboxy-SNARF-1 was monitored following Ca^{2+} changes (**Figure 7**). The mitochondrial pH was not affected by the increase in $[Ca^{2+}]_m$. The resting mitochondrial pH was 7.504 ± 0.047 (mean ± S.E., n = 13). From these results, 5.28 µM was the chosen K_d value of fura-2-FF at pH 7.5.

FIGURE & TABLE LEGENDS:

Figure 1: A microfluorometry system for multiparametric measurement

The schematic diagram of the microfluorometry system was shown. The mounted cells were visualized via a CCD camera. Four different emission lights were detected with four PMTs via a photon counting system. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 2: Identification of isosbestic points

(**A**) The red arrow points to the isosbestic point at the 450 nm emission wavelength. Fura-2 FF in the non-bound state is shown with a dotted line and in the Ca^{2+} bound state with a solid line. (**B**) The red arrow is pointed to the isosbestic point at the 500 nm emission wavelength. (**C**) The subtracted data of the signal at 450 nm in Ca^{2+}-free conditions from Ca^{2+}-free saturated conditions are shown. (**D**) The subtracted data of the signal at 500 nm in Ca^{2+}-free conditions from Ca^{2+}-free saturated conditions are shown. (**E**) Standard deviation data from graph C are shown. (**F**) Standard deviation data from graph D are shown. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 3: Measurement of R_N factors.

(**A**) Changes in the NADH signal without fluorescent dye by applying various mitochondrial substrates were measured at 361 nm excitation and 450 nm emission wavelengths. (**B**) The NADH interference in the fura-2-FF signals, $F_{400,500}$ (⋯) and $F_{353,500}$ (—), were simultaneously monitored. (**C**) The relationships between $F_{361,450,NADH}$ and $F_{400,500,NADH}$ (○)and between $F_{361,450,NADH}$ and $F_{353,500,NADH}$ (●) are shown. The obtained slopes are represented as R_{N1} and R_{N2}, respectively. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 4: Results of NADH and fura-2-FF interference correction

The change of the signals from before the correction (shown in the left panels) to after the correction (shown in the right panels). (**A**) NADH signals at the 450 nm emission wavelength. (**B**) Fura-2-FF signals at the 500 nm emission wavelength. The figure shows $F_{400,500}$ (– –), $F_{353,500}$ (----), and the ratio of fura-2-FF (—). (**C**) The mitochondrial calcium concentration. The red dotted line indicates the zero. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 5: Resting $[Ca^{2+}]_m$ without cytosolic Ca^{2+} and maximal steady state $[Ca^{2+}]_m$ at 1 μM cytosolic Ca^{2+}

Mitochondria were energized with the perfusion of malate-pyruvate solution. The steady state $[Ca^{2+}]_m$ in a Ca^{2+}-free conditions and in 1 μM Ca^{2+} conditions were shown. The addition of 5 mM Na^+ recovered NADH and reduced $[Ca^{2+}]_m$ to the baseline. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 6: Simultaneous measurement of NADH, $[Ca^{2+}]_m$, and Ψ_m

Mitochondria were energized with the perfusion of malate-pyruvate solution. The changes of NAHD, $[Ca^{2+}]_m$ and Ψ_m were shown. The addition of 1 μM Ca^{2+} decreased NAHD and increased $[Ca^{2+}]_m$ but Ψ_m was not changed significantly. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Figure 7: Simultaneous measurement of NADH, $[Ca^{2+}]_m$, and pH

The repeated application of Ca^{2+} could induce the decrease of NADH and the increase of $[Ca^{2+}]_m$ but the mitochondrial pH was not affected by the application of Ca^{2+}. The addition of Na+ could

return the NADH and $[Ca^{2+}]_m$ to the baseline. This figure has been reproduced with permission from The Korean Journal of Physiology & Pharmacology[10].

Table 1: Solutions

DISCUSSION:

The interference correction method was successfully developed for measuring the signals of NADH and fura-2 analogs. Exact measurement of the signals is essential for exact correction. However, the inherent nature of the fluorescent device produces a background signal unrelated to that of NADH of fura-2. The highest quality band-pass filter can only pass up to 10^{-8} of the unwanted wavelengths of the light. However, the fluorescent signal from a single cell is very small, and the reflection of the excitation light after the band-pass filter is still strong enough to contaminate the actual fluorescent signals. Therefore, careful correction of the background signal is necessary.

Fura-2 has a loading problem to measure mitochondrial Ca^{2+}. First, it is not easy to load the dye specifically into the mitochondria, and nonspecific loading into another organelle could be erroneous. Mitochondrial Ca^{2+} concentration is generally higher than that of the cytosol, and the use of fura-2-FF with a high K_d value could avoid the contamination of cytosolic Ca^{2+} changes. The other problematic organelle is the sarcoplasmic reticulum (SR). However, the distribution volume differences (SR 3.5% vs. mitochondria 34%–36% in rat ventricular myocytes)[13,14] and the removal of ATP in experiments could compensate for the contamination from SR.

Our calibration equation (Equation 14 and 15) has many advantageous characteristics over Grynkiewicz's equation[15] as follows:

1) It requires only three parameters: Kd, $F_{400,500,max}/F_{353,500,max}$, and R_{min}.
2) There is linearity of the ratio value to the Ca2+ concentration at a constant pH.
3) There is a relative error-free parameter in $F_{400,500,max}/F_{353,500,max}$ compared with S_{f2}/S_{b2}[15].
4) In Equation 15, only K_d and R_{min} are required if isosbestic excitation is used.
5) The calibration procedure to obtain the parameter is much simpler with Equation 15.

However, there is a limitation because Ca^{2+}-saturated fura-2-FF generates a very small emission. It causes an error. The new equation can be applied to $[Ca^{2+}]$ concentrations up to 50x that of K_d.

In conclusion, a protocol was developed to successfully solve the existing problem of NADH and fura-2-FF interference. This method can measure Ca^{2+} dynamics more accurately. Multiparametric measurement system, particularly in the mitochondria, will help understand the mitochondrial physiology in a quantitative way.

ACKNOWLEDGMENTS:

This work was partially supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1A6A3A01011832), by the Ministry of Science, ICT & Future Planning (2014M3A9D7034366) and by the Ministry of Trade, Industry & Energy (10068076).

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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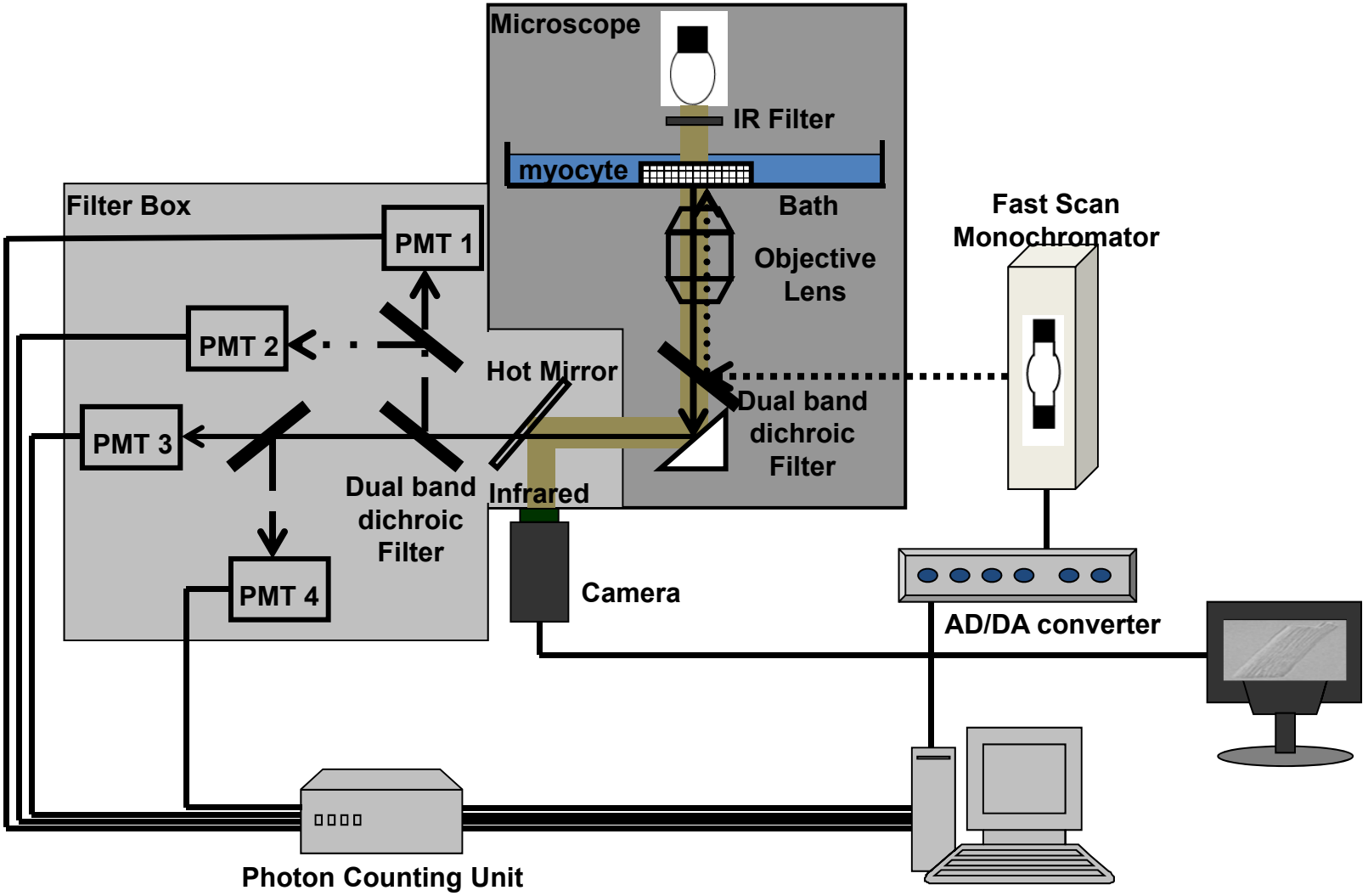
Figure1



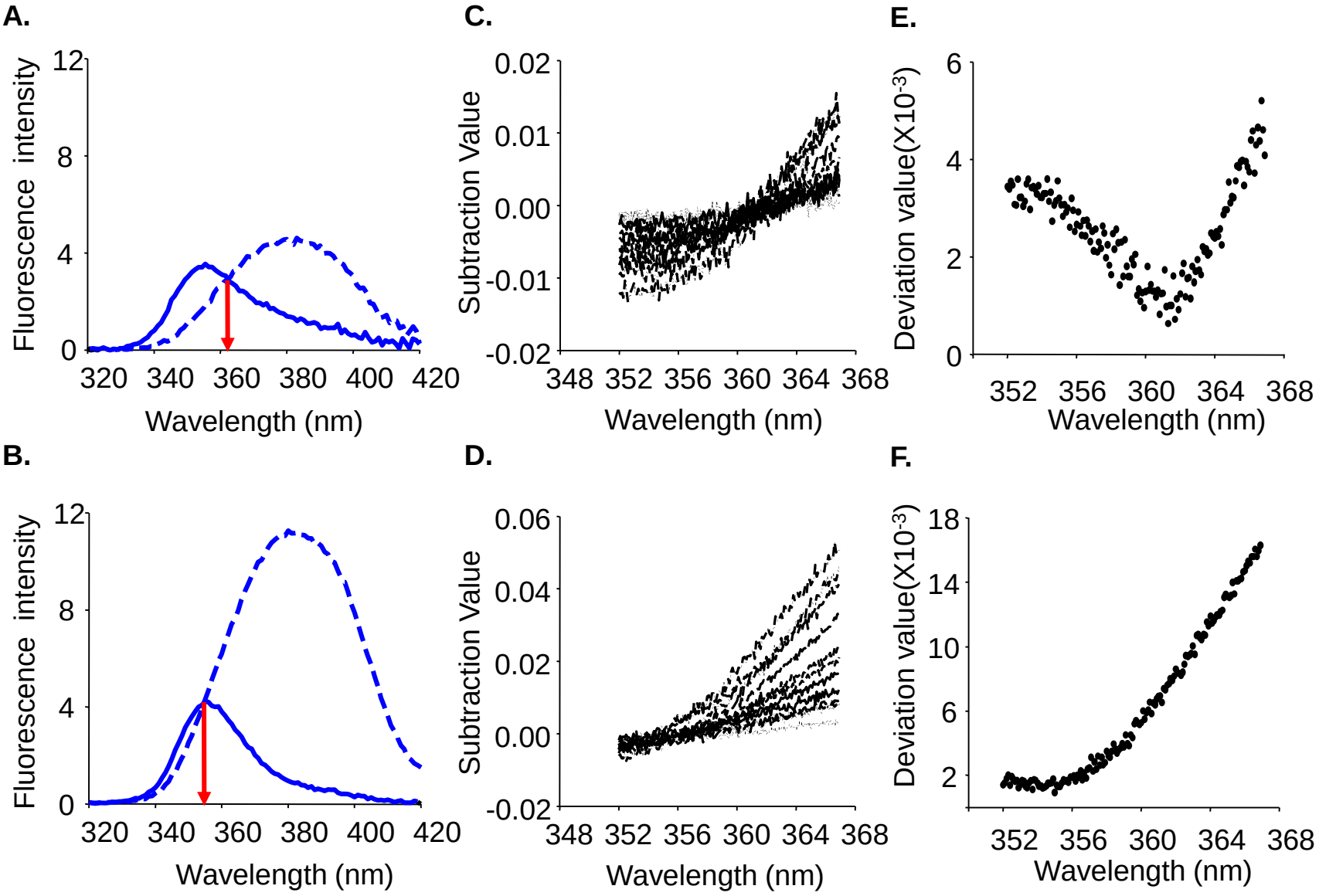


Figure3

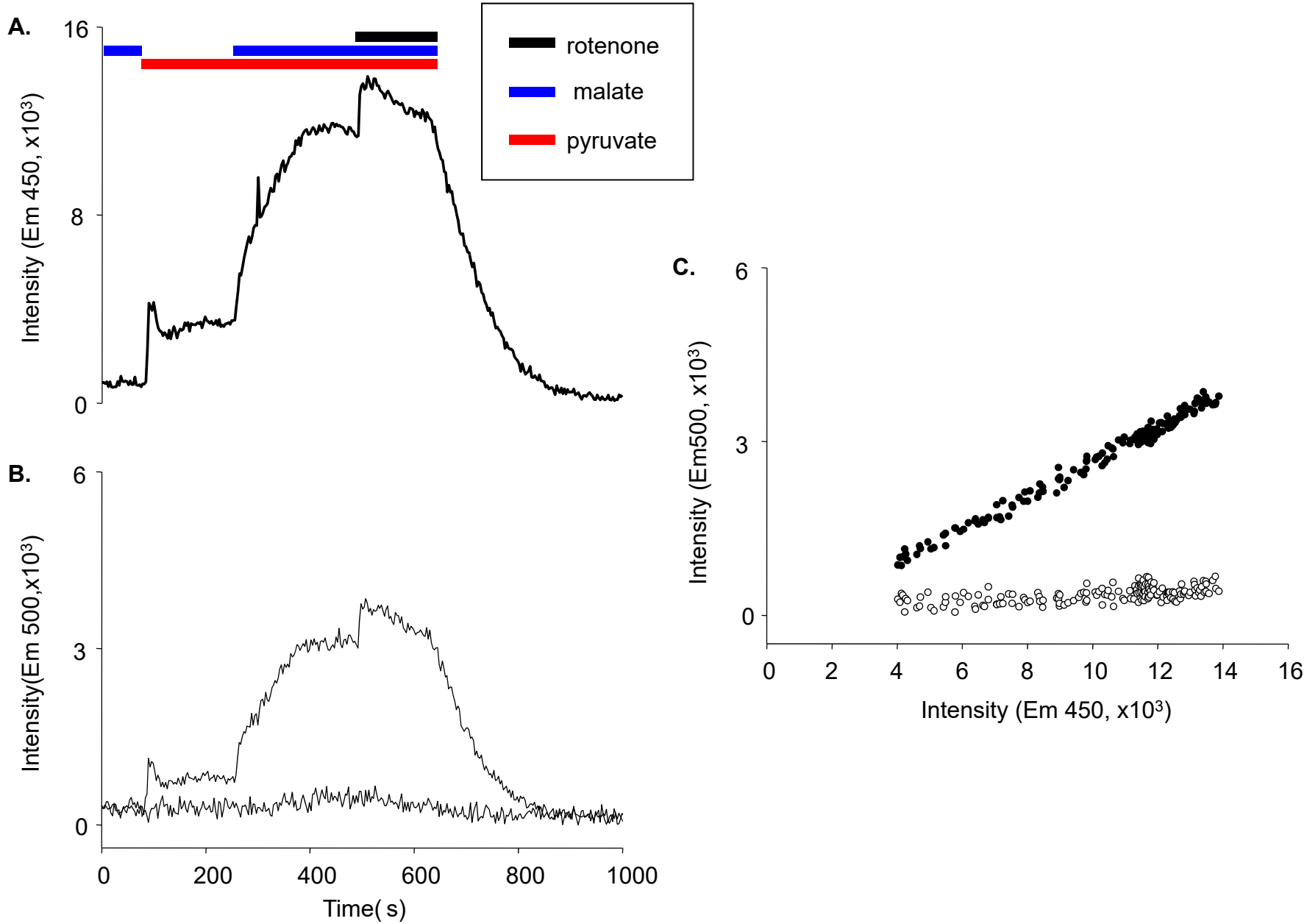


Figure4

Click here to access/download;Figure;figure4_190522.pdf

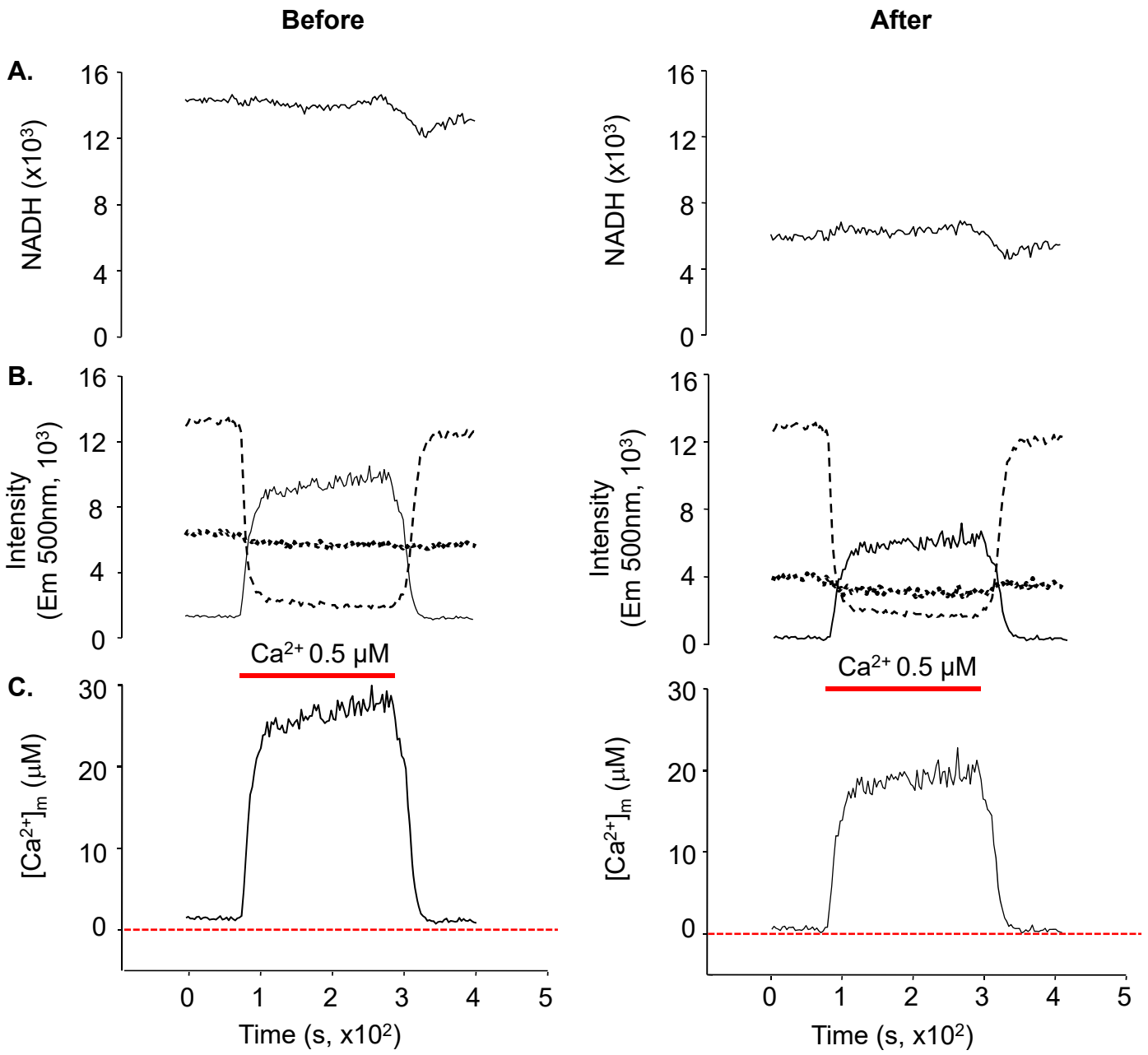


Figure5

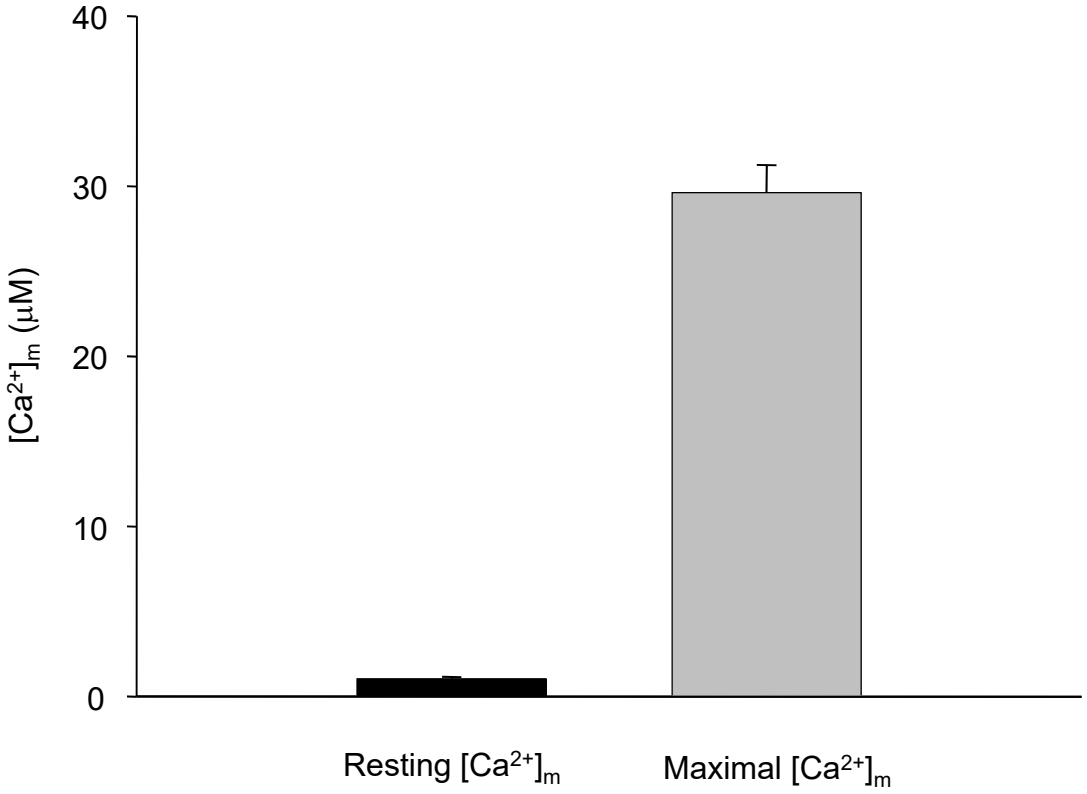


Figure6

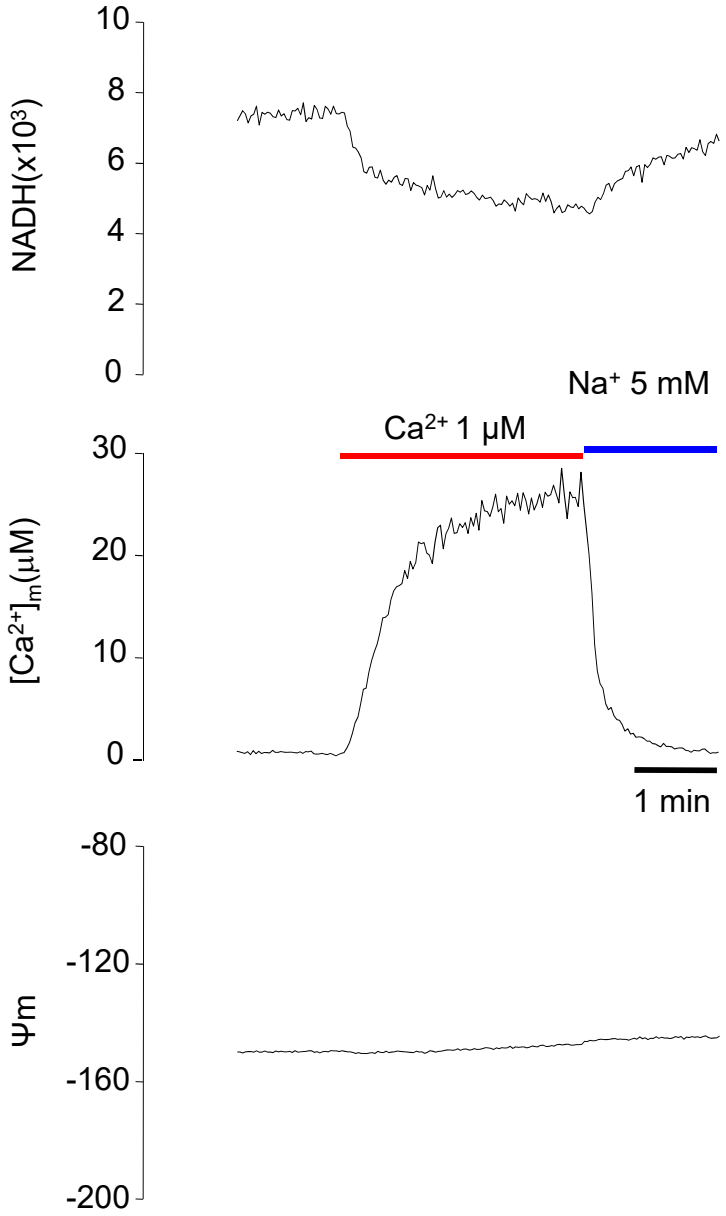
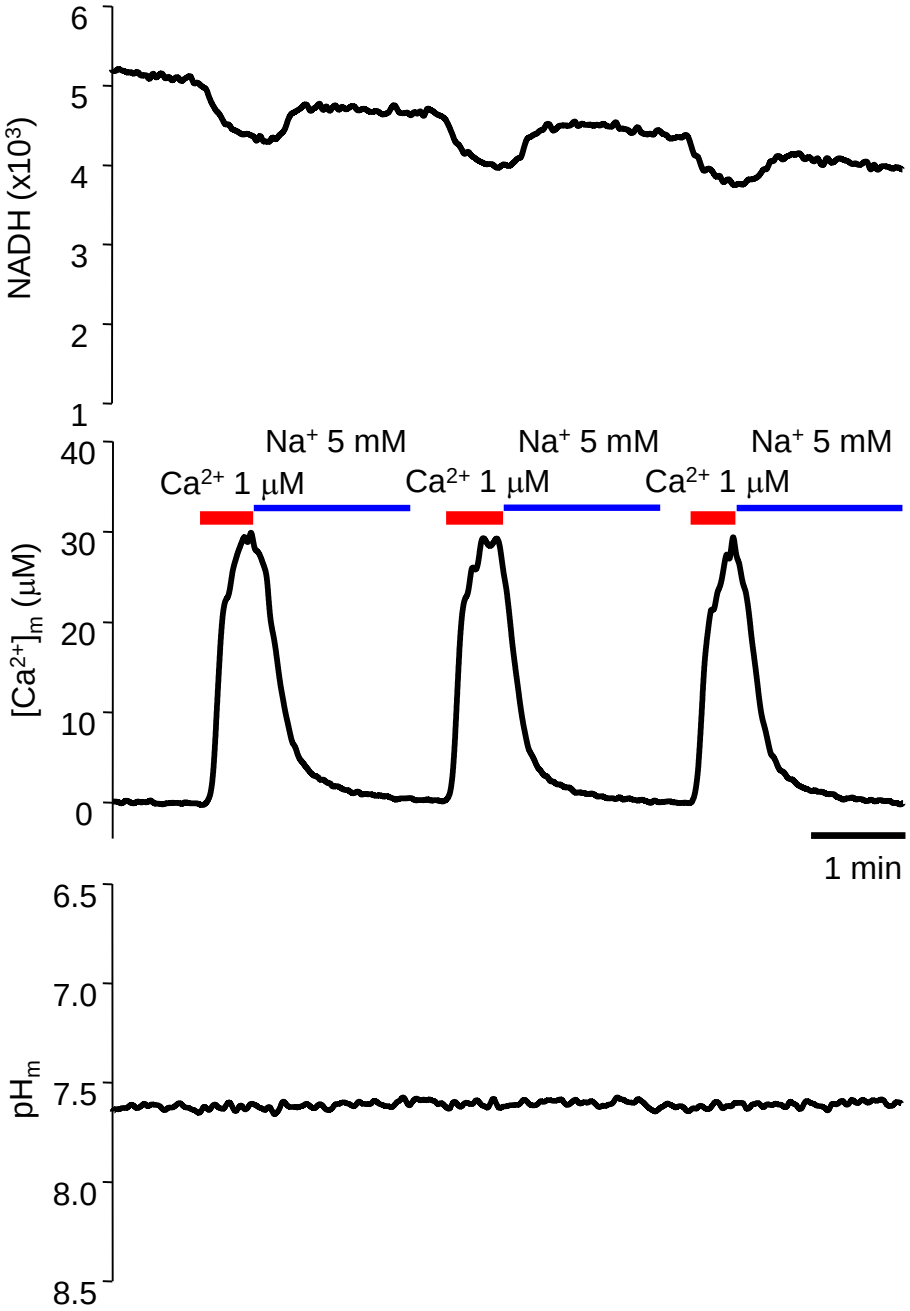


Figure7



Table

Name of Solutions	Concentration (mM)									
	KCl	HEPES	EGTA	$CaCl_2$	M	P	R	FCCP	ADP	Saponin
Ca^{2+}-free	150	10	1							
NADH-free Ca^{2+}-free	150	10	1					0.01	0.1	
NADH-free Ca^{2+}-Saturated	135	10		1				0.01	0.1	
Saponin	150	10	1							0.1mg/ml
Malate	145	10	1		5					
Pyruvate	145	10	1			5				
Malate-pyruvate	140	10	1		5	5				
Rotenone	140	10	1		5	5	0.01			
Culture Medium	Dulbecco's Modified Eagle's Medium (DMEM)									
Dye-loading	Add an 1mM Fura-2-FF-AM stock(16 μL) in the Culture medium (2 mL)									

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2 mL eppendorf tube.	Axygen	MCT-200-C	2 mL Tube
AD/DA converter	Instrutech	ITC-18	Equipment
ADP, Adenosine 5'-diphosphate monopotassium salt dihydrate	Sigma-aldrich	A5285	Chemicals
Band pass filter	Ealing Electro-Optics, Inc	35-3920	Equipment, 640±11nm
Band pass filter	Omega Optical	690-9823	Equipment, 590±15nm
Band pass filter	Omega Optical	500DF20-9916	Equipment, 500±20nm
Band pass filter	Chroma Technology Corp.	60685	Equipment, 450±30nm
Calcium chloride solution	Sigma-aldrich	21114	Chemicals
carboxy-SNARF-1(AM)	Invitrogen	C1272	Chemicals
Charge-coupled device (CCD) camera	Philips	FTM1800NH/HGI	Equipment
Dichroic mirror	Chroma Technology Corp.	86009	Equipment, Multiband dichroic mirror, Reflection : <400nm, 490±10, 560±10, Transmission : 460±15, 510±20, >580nm
Dichroic mirror	Chroma Technology Corp.	567DCXRU	Equipment, Reflection : <560nm, Transmission : > 580 nm
Dichroic mirror	Chroma Technology Corp.	480dclp	Equipment, Reflection : <470nm, Transmission : > 490 nm
Dichroic mirror	Chroma Technology Corp.	20728	Equipment, Multiband dichroic mirror, Reflection : <405nm, 470±30, Transmission : 430nm~520nm, > 640 nm
Dimethyl sulfoxide(DMSO)	Sigma-aldrich	154938	Chemicals
DMEM, Dulbecco's Modified Eagle's Medium	Sigma-aldrich	D5030	Chemicals
EGTA, Egtazic acid, Ethylene-bis(oxyethylenenitrilo)tetraacetic acid, Glycol ether diamine tetraacetic acid	Sigma-aldrich	E4378	Chemicals
FCCP, Mesoxalonitrile 4-trifluoromethoxyphenylhydrazone	Sigma-aldrich	21857	Chemicals
field diaphragm	Nikon	86506	Equipment
Fura-2-FF(AM)	TEFLABS	137	chemicals
Green tube	DWM		test tube
HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)	Sigma-aldrich	H3375	Chemicals
High-speed counter	National Instruments	NI-6022	Equipment
Hot mirror	Chroma Technology Corp.	21002	Equipment, 50:50
Inverted microscope	Nikon	TE-300	Equipment
Malate	Sigma-aldrich	27606	Chemicals
Near infrared filter	Chroma Technology Corp.	D750/100X	Equipment, 750±100nm
Oil immersion lens	Nikon	MRF01400	40x, NA 1.3; Equipment
Photon counter unit	Hamamatsu	C3866	Equipment
Photon multiplier tube	Hamamatsu	R2949	Equipment
Polychrome II	Till Photonics	SA3/MG04	Equipment
Potassium chloride	Merck	1.04936	Chemicals
Potassium hydroxide solution	Sigma-aldrich	P4494	Chemicals
Pyruvate	Sigma-aldrich	107360	Chemicals
Rotenone	Sigma-aldrich	R8875	Chemicals
Saponin	Sigma-aldrich	S4521	Chemicals
TMRE, Tetramethylrhodamine, ethyl ester	Molecular probes	T669	Chemicals

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Author(s):	Jeong Hoon Lee, Jeong Mi Ha, Quynh Mai Ho and Chae Hun Leem

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Editorial comments

The significant role of intracellular Ca^{2+} is widely known and does not need to be explained in detail.

I change as follows and add a reference.
The significant role of intracellular Ca^{2+} is widely known

Steps 1-2 of the protocol can be removed entirely to focus the manuscript on the dye loading and the signal correction. Your suggestion of having a note or one line step is sufficient as long as there is a citation. Tables 1 and 2 should remain in the manuscript. This is to allow others to find the resources to replicate the steps. If steps 1-2 remain in the protocol, additional details will be needed however.

I deleted the previous steps 1 and 2 and changed the preparation of the solution. All solution compositions are displayed in the separate table

As steps 3, 4, and 5 are the most important parts of the protocol and the manuscript, we ask that additional details be provided so others can replicate the protocol with high fidelity.

Such as how to detect the signals and how to get the cell area is the area of the device and the software. Each lab may have different configuration and different software. I just mention the principle to be used. Except the device dependent procedure, I tried to add the explanation in detail.

3.1: What does one stock of fura-2-FF-AM mean?
4.7: Please specify the dichroic mirrors by their characteristics instead of the model number.
5.1.1/5.2.1: How many cells are mounted and at what density?
Please specify the perfusion rate for all perfusion steps.

Please provide a short legend for Figures 1, 5, 6, and 7.

I added and changed as your comments. The solution perfusion rate and the temperature are clearly mentioned in the procedure at the beginning. Appropriate cell density is microscope dependent and therefore, I mentioned it as one cell per one objective field.

I added the legends in Figure 1, 5, 6, and 7.