Light-induced Ca\(^{2+}\) transients observed in widefield epi-fluorescence microscopy of excitable cells

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Abstract: We have investigated the possibility that variations in the level of intracellular Ca\(^{2+}\) in excitable cells might be induced as an artifact of the incoherent illumination that is being used to monitor transient responses. In order to avoid the fluctuations in power of an arc lamp source, a microscope using a light emitting diode that was calibrated accurately at low power levels, was constructed to provide good control over the dose of light applied to the biological specimen. We report here that higher powers of illumination increased the probability of occurrence of Ca\(^{2+}\) transients even in the sub-mW range normally used to measure such transients in epi-fluorescence work, suggesting that caution should be exercised when designing experiments and interpreting data.

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1. Introduction

With innovations in photochemistry such as the development of fluorescent proteins and synthetic biomarkers [1,2], and advances in light emitting diodes (LEDs) [3,4], computers and electronic detectors specifically for imaging applications [5], epi-fluorescence microscopy techniques have become widely used in the study of live biological specimens to examine morphology and physiological processes. Except in experiments where photo-bleaching is deliberately induced (e.g. in order to measure the rate of recovery of fluorescence [6,7]), the levels of light used in widefield epi-fluorescence microscopy are typically a few mW or sub-mW. At these levels, photo-bleaching is slow and it is widely assumed that under these experimental conditions, the incoherent light has no effect on the specimen.

It has been reported previously that laser scanning microscopy can induce physiological responses [8–10]. For example, Smith et al. [8,9] demonstrated that ultra-short pulsed near-infrared lasers similar to those used in multi-photon imaging can evoke intracellular Ca\(^{2+}\) transients, while Knight et al. demonstrated that lower power continuous wave lasers usually employed in confocal microscopy may trigger transient responses and cell death [10]. However, it is not confocal or multi-photon imaging techniques that are most frequently employed in studying Ca\(^{2+}\) transients, but widefield epi-fluorescence microscopy.

We report here that sub-mW levels of widefield incoherent light may also evoke physiological responses in live cells. Isolated smooth muscle cells, excited using an LED to deliver a controlled dose of incoherent radiation, exhibited Ca\(^{2+}\) responses, with the probability of occurrence of transients depending on the illumination power. We show that the widefield illuminating beam cannot be assumed to be without effect on normal cell physiology.

2. Methods

2.1. Cell isolation

Male guinea pigs (500–700g) were sacrificed by cervical dislocation and immediate exsanguination in accordance with the Animal (Scientific Procedures) Act UK 1986. A segment of distal colon (~5 cm) was immediately removed and transferred to an oxygenated (95% O\(_2\)–5% CO\(_2\)) physiological saline solution of the following composition (mM): NaCl 118.4, NaHCO\(_3\) 25, KCl 4.7, NaH\(_2\)PO\(_4\) 1.13, MgCl\(_2\) 1.3, CaCl\(_2\) 2.7 and glucose 11 (pH 7.4). Following the removal of the mucosa from this tissue, single smooth muscle cells, from circular muscle, were isolated using a two-step enzymatic dissociation protocol [11], stored at 4 °C and used the same day. All experiments and loading of cells with fluorescent dyes were conducted at room temperature (20 ± 2 °C). Colonic smooth muscle cells were selected for this study as they are robust cells which allow for imaging over extended periods of time without reduced cell viability.

2.2. Epi-fluorescence microscopy

Cells were loaded with the membrane-permeable dye, fluo-3 acetoxy-methyl ester (AM) (10µM; Molecular Probes, Paisley, UK) for 30 minutes prior to the beginning of the experiment. The composition of the extracellular solution was (mM): Na glutamate 80, NaCl

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40, tetraethylammonium chloride (TEA) 20, MgCl$_2$ 1.1, CaCl$_2$ 3, HEPES 10 and glucose 30 (pH 7.4 adjusted with NaOH 1M). Initially, we used a standard epi-fluorescence microscope (Nikon Eclipse E600 with mercury arc lamp system) but this proved difficult due to fluctuations in average power and so we used a modified microscope to give better control over the illumination. The experimental layout we used for our study is shown in Fig. 1. Cells were placed in a recording chamber (1ml) on the stage of an inverted fluorescence microscope (Olympus IX81). Instead of using the epi-fluorescence illumination system provided by the manufacturer, we adapted the system to couple in an external LED as the excitation source. The system is deliberately simple, providing the same excitation geometry as used in conventional epi-fluorescence microscopy, but with good control over the illumination power and improved detection to capture rapid changes in fluorescence. While the design of the microscope is intrinsically safe due to the low power of the LED light source, great care must be taken if considering using a similar design with more powerful sources such as arc lamps, as blindness could result. The use of safety interlocks in such a scenario is of importance and should be considered.

![Fig. 1. Schematic of widefield epi-fluorescence and brightfield transmission microscope system. Brightfield transmission was collected by the condenser lens and viewed through the eyepiece. White light illumination from the lamp was computer controlled via a flip mirror. The 488 nm LED excitation source was lensed and filtered externally from the inverted microscope to uniformly fill the field of view. Fluorescence was detected using a highly sensitive fast CCD camera with a 520nm LP filter in the detection path.](image)

We used an LED (Luxeon Star K2, Lumileds Lighting) as the excitation source to minimize amplitude fluctuations. The LED had a peak emission wavelength of 488 nm, collimated by single lens (LXHL-NX05, Lumileds), anti-reflection coated for the visible spectral region. An optical bandpass filter (FF01-475/35-25) was used to ensure that only 475 $\pm$ 35 nm output was used for excitation. The LED output power was controlled by a power supply (Iso-tech IPS303A), which provided a maximum current and voltage of 3 Amps and 30 Volts respectively, satisfying the maximum operational parameters suggested by the LED manufacturer.
A 40x/0.9 N.A. dry objective lens (UPlanSApo, Olympus) was used for all experiments providing a field of view of (0.316 mm x 0.316 mm) and a theoretical lateral resolution of 330 nm [12]. The spectral reflector used was a 520 nm long-wave pass filter, reflecting the shorter wavelength excitation towards the specimen and the longer wavelength fluorescence to the detector. The LED filled the field of the objective uniformly with light to illuminate an area of 0.316 μm x 0.316 μm.

The light power and hence intensity delivered to the cells on the microscope stage was controlled by maintaining the LED at fixed voltage and altering the applied current. During this study, four power parameters were used as described in Table 1. The average power at the sample is quoted with a standard scale reading error. The light power was measured using a sensitive photodiode based power meter (Nova II, Ophir Optronics) for each current value applied to the LED. The lowest power of 30 μW was chosen because lower powers of illumination gave a fluorescence signal almost indistinguishable from the background intensity level. As we used a dry objective lens for the study, no immersion material was required for this measurement. The average power was measured before every imaging experiment to ensure the power at the sample did not vary between different experiments. All experiments were undertaken in a dark room environment to minimize light exposure to the sample and also to prevent leakage of room light into the camera.

| Current (A) | Power (μW) | Power per unit area (nW/μm²) |
|------------|------------|------------------------------|
| 0.1        | 30 ± 0.5   | 0.30                         |
| 0.2        | 70 ± 0.5   | 0.70                         |
| 0.3        | 110 ± 0.5  | 1.10                         |
| 0.4        | 150 ± 0.5  | 1.50                         |

For capture of the images and video clips, a charge-coupled device (CCD) camera (I-PentaMAX:HQ, Princeton Instruments) was used at 10 fps. This system was computer controlled using WinFluor software [13] and the same software was used for image acquisition.

Before each imaging session, the CCD camera was pre-cooled to −20 °C for at least ten minutes to reduce dark noise levels. The alignment of the LED was also checked using a standard fluorescent test slide (*Convallaria* specimen, Leica).

To perform the experiment, a cell was brought into the field of view and into focus using brightfield transmission imaging at low power (sub-10 μW). Cells that appeared rounded or showed signs of blebbing or structural damage under brightfield illumination were excluded from our study. The same selection protocol is also used by electrophysiologists working on cells of this type.

With the cell in focus, we then switched to epi-fluorescence excitation and recording using the LED and fast CCD camera. Initially, the current was adjusted to 0.1 A (corresponding to the lowest light power, 30 μW) used in the experiment and a movie was recorded using the fast CCD camera for durations in excess of 60 seconds, during which time the fluorescence signal was recorded. After each movie, a different cell was selected. Cells were selected systematically by moving the recording chamber in one direction to ensure that the same cells were not re-exposed to the visible wavelength excitation source. This process was repeated at the power levels detailed in Table 1 for n = 30 cells per power level.

The movie files generated were then analyzed using the same software as for movie acquisition (WinFluor). Region of interest (ROI) analysis of the fluorescence signal intensity of the full or partial cell was performed over the duration of the movie. A localized response was defined as an increase in fluorescence signal intensity that exceeded 10% of the baseline, but which occurred over less than 50% of the cell body, while a global response was defined as a fluorescence signal intensity that exceeded 10% of the baseline level and occurred over more than 50% of the cell body. A null response was taken as a fluorescence signal intensity
which did not vary by >10% from the baseline fluorescence level. To verify the significance of the results observed, $\chi^2$ testing was performed.

3. Results

To investigate the effects of low light intensity illumination during epi-fluorescence microscopy on the number of cells exhibiting Ca$^{2+}$ responses, cells were loaded with the fluorescent Ca$^{2+}$ indicator fluo-3 AM and exposed to varying illumination powers for at least 60 seconds. Ca$^{2+}$ responses, measured as changes in fluorescence intensity were analyzed and spatially categorized as described previously (see methods).

Few responses were observed at low incident powers. However, the number of cells exhibiting a Ca$^{2+}$ response increased with increasing excitation powers. Cells exhibiting Ca$^{2+}$ responses were subdivided into those exhibiting either a local or global Ca$^{2+}$ rise. The number of cells exhibiting each response at incrementally increasing illumination power is illustrated in Fig. 2. The cells that did not respond are not shown explicitly. The results are presented with a 5% error in detection of a fluorescence signal increase (local or global) and with a standard error for optical power. At 30 μW power, only a minority (10%) of cells exhibited Ca$^{2+}$ responses. Of these, 7% exhibited a local response (2 of 30 cells) and 3% exhibited a global response (1 of 30 cells). At increasing excitation powers, the number of cells exhibiting Ca$^{2+}$ responses increased to 60% and 43% at 110 μW and 150μW, respectively. At 110 μW, 36% of cells exhibited a local response (11 of 30 cells) and 23% of cells exhibited a global response (7 of 30 cells). Increasing the power to 150μW did not further increase the percentage of cells exhibiting either a local (33%; 10 of 30 cells) or global rise in Ca$^{2+}$ (10%; 3 of 30 cells). An unexpected decrease in the number of Ca$^{2+}$ responses at 150 μW in comparison to illumination at 110 μW was observed (Fig. 2). However, from $\chi^2$ tests this was not statistically significant ($p>0.05$).

![Fig. 2](image.png)

Fig. 2. The number of cells exhibiting Ca$^{2+}$ responses for $n = 30$ cells are shown for each optical power setting. The responses are categorized as global or localized response. The data is expressed with a 5% error in detection for optical power.

Representative examples of localized and global Ca$^{2+}$ responses at 150 μW power are illustrated in Fig. 3. Figure 3(a) shows a localized Ca$^{2+}$ rise at t = 16.4 seconds. In the same cell, a global Ca$^{2+}$ transient was observed (Fig. 3(b)) following the local response (t = 36.8 seconds). The corresponding localized and global Ca$^{2+}$ traces are illustrated in Fig. 3(c).

A representative frame from a video clip demonstrating a single global Ca$^{2+}$ transient is shown in Fig. 4 (Media 1). Here, for illustration purposes, the movie is truncated from t = 0 seconds to t = 80 seconds, where 30 μW average power was applied to the specimen, during which time the cell was brought into focus and centered on the field of view. The movie is presented from t = 80 seconds that continues illumination with 30 μW average power. At t =
164 seconds, the average power applied to the specimen was increased from 30 μW to 70 μW, and the power was again increased, from 70 μW to 110 μW at t = 271.2 seconds. The movie is shown together with the fluorescence signal intensity plot, where the signal is monitored at a single point, marked on the cell body (+). The movie capture rate of 10 Hz is indicated on the y-axis (10). At 30 μW and 70 μW powers, no apparent changes in fluorescence signal intensity were observed, relative to the proportional increase in background signal. However, at t = 307.6 seconds (110 μW), a global Ca²⁺ transient was evoked, with several orders of magnitude increase in fluorescence signal that returned to baseline. A rise in intracellular Ca²⁺ concentration triggers cellular contraction (as shown in (Media 1)) and can cause cell death. However, the ability of each cell to relax following contraction suggests that the powers of illumination used in this study, including higher powers, did not cause irreversible cell damage (n = 30).
A global Ca\textsuperscript{2+} transient was observed after a step-wise increase in optical power (Media 1). The specimen was initially irradiated from \(t = 0\) to 80 seconds at 30\(\mu\)W (not shown). The 30 \(\mu\)W irradiation continued from \(t = 80\) seconds until \(t = 164\) seconds, when the average power was increased to 70 \(\mu\)W. The specimen was illuminated from \(t = 164\) seconds to \(t = 271.2\) seconds, and then the power was incremented to 110 \(\mu\)W for the remainder of the experiment. A global Ca\textsuperscript{2+} transient is observed with illumination of 110 \(\mu\)W average power at \(t = 307.6\) seconds. The selected frame shows the recovered cell at \(t = 400.0\) seconds. The cross and ‘1’ indicates a highlighted single point from which the fluorescence intensity over time data was plotted.

The representative images in Fig. 5 illustrate contraction and recovery of a smooth muscle cell following a global rise in Ca\textsuperscript{2+}. Figure 5(a) shows a cell in which no Ca\textsuperscript{2+} responses were observed under 70 \(\mu\)W illumination. Increasing the illumination power to 110 \(\mu\)W evoked a global rise in Ca\textsuperscript{2+} (Fig. 5(b)) resulting in cellular contraction (Fig. 5(c)) and recovery (Fig. 5(d)). Again, no morphological changes were observed that would indicate cell damage.

4. Discussion

Intracellular Ca\textsuperscript{2+} is a ubiquitous second messenger controlling fundamental and diverse cellular processes, including cell division, growth and cell death and provides the major trigger for smooth muscle contraction. Here, we demonstrate an increase in the number of cells exhibiting Ca\textsuperscript{2+} responses at increasing excitation power of the light source, which is marked even in the sub-mW range. Thus, light-induced Ca\textsuperscript{2+} signalling may trigger alterations in numerous cell processes at power levels provided by standard, commercial epi-fluorescence microscopes. Caution should be exercised in interpreting data.
There has been little work to date on disturbances in cell physiology at the low powers of incoherent light used here. This is possibly because the mercury arc lamp used as a source in many epi-fluorescence studies shows marked fluctuations such as those observed by us in our experimental design process, making quantification of the type done here difficult [14]. Our use of an LED as the excitation source overcomes this limitation and has allowed investigations with low powers of incoherent light. However, it is also possible that in standard epi-fluorescence systems using an arc lamp as the excitation source, the average power at the specimen can be much greater than that used in our study. With a FITC cube to provide a similar excitation wavelength range in the Nikon microscope and arc lamp system we initially investigated, the average power at the sample plane was measured to be 2.04 mW with no attenuation of the excitation source, and 529 μW for ND = 4, 230 μW for ND = 8 and 73.3 μW for ND = 12. We note that all of these powers exceed our lowest applied power of 30 μW, further validating our choice of 30 μW for low power illumination. Our observation of an increased number of Ca²⁺ transients occurring at higher average excitation power therefore leads us to suggest that regardless of the light source used, whether LED or arc lamp, in practical live cell physiological imaging the applied power should be kept as low as possible and the light source may require attenuation beyond the standard options provided on commercial instruments.

The underlying mechanism(s) mediating photo-induced Ca²⁺ signalling remain unclear [15]. However, the Ca²⁺ transients observed in our study are unlikely to be attributed to warming as a consequence of water absorption, as the temperature rise would be insignificant at the powers used in our study [16]. Neither can the presence of TEA alone explain these results. While we cannot fully exclude any influence of TEA present in the extracellular solution bathing the cells on either the generation of Ca²⁺ transients or the sensitivity of the cells to light, photo-induced Ca²⁺ signaling is observed in single cells and intact tissue in the absence of TEA or a photosensitizing agent [8–10]. Nevertheless, we advise caution when TEA is included in the bath solution.

Absorption of the visible light by a constituent of the cell, as demonstrated in motor end plates [17], the applied fluorochromes or colored pigment [18] is more likely. Yet, light-induced effects are unlikely to be specific to the use of fluo 3-AM in this study because photo effects are also observed in cells stained with various fluorochromes, including fluo 4 [8–10]. Indeed, light-induced release of reactive oxygen species (ROS), associated with the interaction of light and the fluorochrome, has been shown to evoke Ca²⁺ responses and cell death [10]. Although such chemically reactive molecules occur naturally in living cells, and have key roles in cell signaling and homeostasis [19], significant rises in ROS can be detrimental to normal cell function [20]. However, few studies of light-induced ROS with incoherent sources exist. We note the work of Dixit and Cyr [21] who have used both arc lamps and laser systems for studying light-induced ROS, however the light intensities are expressed as percentages of the original source rather than in convenient S.I. units, and so relating their findings to our own work is not possible, particularly given the fluctuations in arc lamp intensity described previously.

5. Conclusion

The present study demonstrates for the first time that low powers of incoherent light may evoke intracellular Ca²⁺ transients, with an increased probability of responses occurring at higher excitation powers. Thus, caution must be exercised in using epi-fluorescence for studies of live cell physiology, even at the mW and sub-mW optical powers commonly used. Even with an incoherent source not brought to a diffraction-limited focus at the specimen it would appear that light is more invasive than is generally thought.

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