UV Ratiometric Imaging Of Isolated Ventricular Cardiomyocytes Using An LED Based Illuminator

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Introduction
Ratiometric fluorescence microscopy methods allow researchers to obtain calibrated images of dynamic changes in the physical properties of cells and tissues independent of dye concentration. The wavelength changes required for ratiometric imaging are routinely achieved using a short-arc source in combination either with a diffraction-grating or interference filters mounted in a filter wheel. Filter wheels typically switch positions in around 50ms, so scanning monochromators can achieve wavelength changes within a ms. These approaches generally limit ratio imaging rates to a little over 10Hz, and have the inherent drawbacks of short lamp life and high thermal emissions. An LED system has much higher stability than a short-arc source, and with sub-microsecond wavelength switching times allows the very high speeds to obtain images that would be impossible with a traditional point source that is typically used for Ar + laser or a xenon arc lamp.

System configuration for 100Hz imaging
For the high speed image acquisition, a back-illuminated multi-channel called the SPEX 1000 by SPEX, is used. It is capable of imaging 3000 channels in parallel using a 30ms exposure time. The camera is configured to trigger off the camera frame sync pulse, and the LED control pulses timed to ensure the LED switched off just prior to the end of the exposure window. This was to ensure the preparation was not illuminated during the period the image is shifted under the mask, and eliminate any potential for smearing of the image.

To obtain clean images the synchronisation of the camera exposure with the illuminator and timing of the exposure is critical. The timing of the LED pulse in different sets of experiments using 100Hz imaging was measured for LED systems using a wide range of excitation wavelengths, and confirmed that the LED pulse duration was about 100µs, and this was repeated in other experiments with different excitation wavelengths.

Methods
Conventional Fura-2 ratios are calculated by monitoring the fluorescence signal elicited by excitation at 340 and 380nm. However, two-wavelength LED systems are capable of exciting the dye at 340nm, and monitoring the fluorescence at 380nm. Alternatively, an LED system designed specifically to excite Fura-2 at 340nm can be used to generate a ratio of 350/380nm. The LED system is capable of exciting the dye at 340nm with appropriate pixel binning, and was cooled to -80°C sufficiently sensitive to acquire a measurable signal in the intervals required.

Fluorescence measurement schematic
To show the spread of the wave through the cell a series of vertical regions, 6 pixels wide, were selected. The mean value for the region was taken at each time point and plotted. The upper trace shows the calcium ratio at the right hand edge of the cell, the lower trace that from the bottom of the cell.

Results
The initial series of measurements were performed using the photomultiplier detector configuration to verify that Calcium ratios obtained from excitation of Fura-2 at 350nm and 380nm gave the same results as those obtained from a standard 340/380nm illumination protocol. For this comparison we first used the Optiscan monochromator equipped with a Xenon arc lamp and recorded the Fura-2 emission using a photomultiplier; we then swapped to the LED illuminator and using the same cells.

Fluorescence images are analysed off-line to generate plots equivalent to those recorded from a ratiometric calcium data acquisition hardware. The region containing the cell was defined and the average signal intensity across this region extracted using a standard ratiometric analysis system. The ratio image was then recorded with a decaying signal level as the dye bleaches. The 350nm signal tracks the change in calcium concentration and the 380nm signal the inverse. The traces shown in the figure above were recorded at 167Hz and normalised to a trace is a mean of 8 traces, and the 380nm signal the inverse. The traces are very fast, and to accurately resolve the transients requires data sampling at speeds of 100Hz or higher. This gives a time window of 10ms to expose and read the images for each ratio pair, or 5ms per image. Acquisition at these short integration times demands the use of the most sensitive detectors available, with precise synchronisation with the light source. To capture images in excess of 100Hz, the camera sensor needs to be binned & a sub-region selected to optimise the system for speed over spatial resolution.

Fluorescent Measurements
Two sets of experiments were carried out, the first to compare the performance of the LED illuminator with a standard Xe arc lamp coupled through a scanning monochromator (Cairn Optiscan). The second was to establish the feasibility of acquiring ratio pairs in the 0.1kHz time domain at 64x32 pixel spatial resolution. In both cases cells were paced at 1Hz using a digitimeter stimulator unit.

System configuration for photomultiplier detection
To compare the LED illuminator with a regular Xenon arc illumination system, a high speed photon counting based detection system was used. Fluorescence excitation was provided by either a Cairn Optiscan monochromator (configured to switch between 340nm and 380nm with a 10nm bandwidth every 6ms) or the Cairn OptoLED illuminator alternating power between two Nichia UV LEDs. The short wavelength was provided by a NC51033A 365nm LED fitted with a D350x filter (Chroma); the long wavelength by a NC51032A 385nm LED fitted with a D385x filter (Chroma). The monochromator was capable of switching wavelengths in under 1ms and unequal illumination intensities used to balance the excitation intensity at the two wavelengths (4ms at 340nm and 2ms at 380nm). To balance the fluorescence excitation of the LED system the powers at the two wavelengths were adjusted. The Fura-2 microscale filter cube consisted of a 400nm long pass dichroic mirror with an emission filter with passband 470-550nm (Chroma).

Cell preparation
Animals were euthanized by administration of an intravenous injection of 500 µL heparin, together with an overdose of sodium pentobarbitone (100 mg kg⁻¹). Heart were removed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Ventricular myocytes were isolated from Langendorff-perfused rabbit hearts using the enzymatic digestion method described previously. Isolated cells were maintained in a modified Krebs solution with no addition of CaCl₂. The superfusate solution used for the experiments contained, in mM: NaCl, 144; KCl, 3; CaCl₂, 1.8; MgCl₂, 1; Hepes, 5; Glucose, 11.1 and CaCl₂, 1.8. All chechrals were obtained from Sigma with the exception of Fura-2 AM and DMSO which were obtained from Molecular Probes and Fluka, respectively.

Results
The traces shown in the figure above were recorded at 167Hz and normalised to a trace is a mean of 8 traces, and the 380nm signal the inverse. This will result in a limited fluorescence signal due to the dye bleaching (clearly evident in the raw traces of area 3). The 350nm signal tracks the change in calcium concentration and the 380nm signal the inverse. The traces shown in the figure above were recorded at 167Hz and normalised to a trace is a mean of 8 traces, and the 380nm signal the inverse. We have used a 512x512 pixel Xenon D9075 in frame transfer mode to monitor calcium transients within a cardiomyocyte while it was externally paced at 1 Hz. These transients are very fast, and to accurately resolve the transients requires data sampling at speeds of 100Hz or higher. This gives a time window of 10ms to expose and read the images for each ratio pair, or 5ms per image. Acquisition at these short integration times demands the use of the most sensitive detectors available, with precise synchronisation with the light source. To capture images in excess of 100Hz, the camera sensor needs to be binned & a sub-region selected to optimise the system for speed over spatial resolution.

Discussion
We show here that our LED system is a suitable alternative to conventional arc lamp sources for ratiometric UV measurements. Furthermore, when used with an EMCCD camera, quantitative spatial information can be recorded with a signal-to-noise ratio suitable for physiologically relevant events, such as calcium waves, to be monitored. The very rapid on/off times of the LED system allow relatively straightforward integration into the imaging system, with alternate frames being clearly illuminated by the selected wavelength. There is an additional benefit whereby careful control of the triggering ensures the illumination is off during the transfer of the image from the light sensitive to the masked region of the sensor, thereby completely eliminating “smearing” of the image that is commonly observed when running frame transfer camera at high speed. High speed multi-wavelength imaging has previously required specialist, often bespoke, instrumentation requiring a significant degree of technical knowledge to use and expensive and the commercial costs. With the OptoLED controller from Cairn coupled with the Xon D9075 from Andor to construct an economical UV ratio measurement system that will allow images to be acquired in excess of 100Hz. With OptoLED camera switching times of sub microsecond, the limiting factor in these experiments was the frame readout and sensor sensitivity. Other camera systems are available that will allow faster rates to be achieved, and together with Cairn’s Optosync timer unit it is now feasible to achieve multi-wavelength imaging at 100’s of Hz with standard commercial products.

Developments in the intensity of power LEDs, the range of wavelengths available are occurring rapidly, so it is likely that in the near future emitters closer to the ideal of 340nm will become available. The ease of control, stability and reproducibility of LED based sources will make these the illuminator of choice for many fluorescence applications in the coming years.

Spontaneous Calcium wave propagating through cell
In this cell a spontaneous calcium wave was observed. Ratio images were acquired at 100’s of Hz, the sequences of images show that the wave propagated through the cell.