High-Speed Recording of Calcium

Ultra-low light calcium signaling can be measured at high speeds.

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Investigating the dynamic balances of ion fluxes, electrical potentials, and pH is crucial for understanding fundamental functions within living cells. Imaging is a common technique to study these vital parameters. The simultaneous measurement of fluorescent signals and cell shape contraction however remains challenging due to different wavelengths or alignment of light paths. This article describes the advantages of using the W-View Gemini image splitting device to observe brightfield and fluorescence signals simultaneously at a very high acquisition rate.

Imaging is widely used to study changes in cellular ionic concentrations, voltage, and pH in life sciences research. Indicators emitting fluorescent light while reacting with the substrate of interest, are often used for indirect visualization of such fluxes. Calcium (Ca$^{2+}$) is important for many physiological processes, including enzyme function, nerve and muscle excitation, or contraction of skeletal and cardiac muscles. Studying the diverse roles of Ca$^{2+}$ has been, and continues to be, heavily reliant upon imaging. New mechanistic insights are possible due to the steady improvement in the detection of Ca$^{2+}$ levels with greater sensitivity, spatial, and temporal resolution.

In cardiac muscle cells (cardiomyocytes), Ca$^{2+}$ imaging has provided critical insight into the process of excitation-contraction coupling and its alterations during disease. This process begins with depolarization of the cell membrane during the action potential and the opening of voltage-gated L-type Ca$^{2+}$ channels. In healthy cells, the resulting Ca$^{2+}$ influx efficiently triggers additional release of Ca$^{2+}$ from the sarcoplasmic reticulum and results in contraction. Excitation-contraction coupling becomes impaired under conditions such as heart failure [1, 2]. The membrane invaginations (t-tubules) that contain L-type Ca$^{2+}$ channels become disorganized and lost in failing cells which reduces the ability of Ca$^{2+}$ influx to trigger Ca$^{2+}$ release [3–5]. The release channels themselves are also suggested to show disrupted organization. And all this together promote a de-synchronized pattern of Ca$^{2+}$ release across the cell [6]. Less efficient Ca$^{2+}$-induced Ca$^{2+}$ release has been previously linked to reduced and slowed contraction of cardiomyocytes, as well as impaired contractility of the heart [7, 8]. However, the precise nature of this correlation is not understood yet, and directly linking Ca$^{2+}$ levels or Ca$^{2+}$ fluxes with cardiomyocyte contraction is not straightforward. Traditionally these measurements have been conducted separately, with Ca$^{2+}$ transients recorded by wide-field imaging, and cell length recorded by edge detection of brightfield images. Such approaches are often hampered by low temporal resolution of the recording, inaccurate edge detection technology, and difficulty with precise post-hoc synchronization of these measurements.

In this article, we demonstrate that an image splitting device mounted to a sCMOS camera enables the simultaneous visualization of such fluxes.
of brightfield and fluorescent signals with high speed and high sensitivity. This technique is employed to concurrently observe calcium release and contraction in cardiomyocytes, but can also be employed in other scenarios with similar imaging demands.

**Experimental Setup**

This section highlights the advantages of the combination of an image splitter (W-View, Hamamatsu Photonics) and an ultra-sensitive and high speed camera (ORCA Flash 4.0 V2, Hamamatsu Photonics) to image calcium signals from cardiac myocytes (provided by William Louch, University of Oslo). The experiment was performed using a chamber mounted on the stage of an inverted Zeiss Observer microscope (Zeiss GmbH, Jena, Germany). Fluorescence excitation light (480 nm) was coupled to the side port of the microscope and directed to the experimental chamber via a dichroic mirror (<510 nm). The chamber was additionally illuminated with brightfield light filtered by an ionic red filter. Emitted fluorescent and transmitted light were passed through the dichroic mirror and then a long pass filter (>515 nm). Collected light was guided to the W-View Gemini by the standard C-mount camera port of the microscope and split by another dichroic mirror (560 nm).

Thus, the fluorescence signal from 515 to 560 nm and brightfield signals >560 nm were separated onto the two sensor halves of a sCMOS camera (Hamamatsu ORCA-Flash 4.0 V2). A scheme of the setup can be seen in Fig. 1. The combination of an image splitter and a sCMOS camera enables the two light signals to be recorded simultaneously. The orientation of the image splitter (blue dashed line) and the camera readout (red line) are shown in Fig. 2a.

The experiment was performed on freshly isolated ventricular cardiomyocytes (see [9] for proto-

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![Image](image.png)

**Fig. 2** a) Orientation of image splitter and readout direction of the sCMOS camera. To achieve high frame rates with low rolling shutter offset the image was not split onto the two sensor halves with its readout registers, but was instead split perpendicularly. This maintains a large field of view (FOV) and fast acquisition times for the two simultaneous signals. b) Raw fluorescence and brightfield images of a cardiomyocyte during the experiment. c) Timing diagram of the experiment. The exposure time was set to 3 ms and the rolling shutter offset was 2.49 ms, as the two readout registers processed 256 lines each (FOV: 2048 × 512 pixels). The clock rate was 9.74 μs/line.
Both measurements are normalized to starting, pre-stimulus Ca\textsuperscript{2+} at room temperature. Ca\textsuperscript{2+} transients were elicited by field stimulation via two platinum electrodes at a frequency of 1 Hz. Raw fluorescence and brightfield images (sized 2048 x 256 pixels) are shown in Fig. 2b.

Recent sCMOS camera technology can image 4 million pixels at 100 Hz. This is achieved by two readout registers which read the horizontal lines of the sensor from the middle to the top and the bottom at once (dual rolling shutter). It is possible to further increase the maximum framerate of the camera by reducing the number of horizontal lines to be read (maximum: 25,655 fps). The timing of the camera, as it was set in the experiment, can be seen in Fig. 2c. Theoretically, a resolution of 2048 x 256 pixels allows for framerates of 800 Hz, but demands exposure times of less than 1.25 ms. At these short integration times, the signal to noise ratio was insufficient, but increasing the exposure time to 3 ms yielded excellent results. This approach was combined with a smaller readout area which reduced the time shift of the top and bottom sensor line from the center line (Trolling = 2.49 ms).

**Results**

Here we demonstrate that both calcium release and cellular contraction can be imaged at 333 Hz. Fig. 3 shows an overlay of the Ca\textsuperscript{2+} signal and cell contraction at four different time points: (a) before stimulation, (b) at peak of Ca\textsuperscript{2+} transient, (c) at peak contraction, and (d) following re-relaxation of the cell. The superior signal to noise ratio of both the fluorescence signal and the transmitted light provides a sensitive, quantitative assessment of this process. Spatially-averaged traces of calcium release and cell contraction are provided in Fig. 4. Cell contraction data was extracted from the images by thresholding the transmitted light signal (> 560 nm), to create an outline of the cell for definition of the ROI. Averaged pixel intensity for the Ca\textsuperscript{2+} fluorescence signal (510 – 550 nm) was normalized to resting fluorescence prior to electrical stimulation. Cell length was measured as the time-dependent length of the thresholded brightfield image. As expected, the rise and fall of transient Ca\textsuperscript{2+} was observed to precede contraction and relaxation of the cell respectively.

**Conclusion**

Mounting an image splitting device on the latest generation sCMOS camera enables dual-channel recording at impressive sensitivity and speed. The ability to simultaneously record transmitted and emitted fluorescent light under such conditions allows for the examination of excitation-contraction coupling in muscle cells and would be similarly well-suited for investigations of fast signaling processes in most biological systems. It was demonstrated that the power of an image splitting device (W-VIEW, Hamamatsu Photonics) mounted to an ultra-sensitive (readout noise < 1 e- ) and high-speed (100 fps @ full sensor readout) camera (ORCA Flash 4.0 V2, Hamamatsu Photonics) mounted to any standard light microscope allows for imaging and measuring fast biological processes simultaneously.

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