Microprocessor-Controlled Laser Scanner System for Multiwavelength Cardiac Optical Mapping

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Abstract

We developed a new laser scanner system that is capable of recording fluorescence spectra at a rapid rate (1 kHz) from 128 laser spots on the heart. The recording of multiple wavelength bands allow the use of emission ratiometry to reduce motion artifact. Original laser scanners were built with control circuitry external to the computer to obtain sufficient speed. Our system eliminates external circuitry by using a 160 MHz microprocessor that controls deflection of the laser beam by Bragg cells and real-time sampling of fluorescence.

Trials were done with six rabbit hearts. Two photomultiplier tubes (PMTs) with a beam splitter sampled green and red fluorescence at 510-570nm and >590nm. Simultaneously, two additional digitizers sampled the laser intensity and stimulation current. As many as eight PMTs may be used to record spectra with greater detail. Sufficient dF/F resolution was obtained to study action potentials.

1. Introduction

Fluorescent dyes that are sensitive to transmembrane potential are used in optical mapping systems to study cardiac action potentials [1]. By recording multiple wavelength bands of fluorescence, emission ratiometry may be employed to reduce the effects of heart motion [2]. Laser scanners are advantageous for the ratiometry because localization is determined by a single laser beam and does not require the alignment of light detection apparatus such as cameras. We designed a laser scanner system driven by a microprocessor that is able to collect up to eight bands of fluorescence while scanning 128 spots per millisecond. Experiments done with isolated perfused rabbit hearts verify the system's ability to study action potentials with transmembrane voltage sensitive fluorescent dye.

2. Methods

In designing the laser scanner system, we wished to upgrade the computer system and much of the digital hardware that was external to the computer in earlier systems. Also, previous systems were able to record for limited duration. We needed the new system to record longer runs. Also, we needed pre-triggering capability in order to allow the recording of cardiac arrhythmias after their visual detection from an ECG monitor.

There were several challenges in designing a system to meet these criteria. The laser beam must change position frequently in order to map 128 spots in 1 millisecond. This produces a 7.8 microsecond spot interval which defines the time interval available to sample fluorescence from any one spot. Sampling must be at an appropriate time within the spot interval.

Also, handling and storage of data are important for laser scanner systems. A previous system used only one photomultiplier tube and recorded for 2.048 seconds with 8-bit resolution at 64 spots. We wanted our system to record from up to eight photomultiplier tubes for up to 14 seconds with 16-bit resolution at 128 spots, producing over 200 times more bytes of data compared with earlier systems.

2.1. Equipment

The microprocessor chosen for the laser scanner system is Innovative Integration's M67 board, a 160 MHz microprocessor containing Texas Instrument's TMS320C6701 integrated circuit. The board plugs into the PCI slot of a 1GHz Dell Dimension 4100 desktop computer (host) and operates independently of the computer. Windows-based systems were considered for the project but were not used due to the strict timing requirements of the laser scanner and the variability of the Windows operating system timing. The M67 board supplies 32 bits of programmable digital I/O. An A4D4 module plugs into the M67 board to provide four digitizers for simultaneous 16-bit 200 kHz A/D and D/A. Another module provides four additional A/D digitizers.

2.2. Software

Two programs were written to operate the microprocessor. The target program was created in Texas

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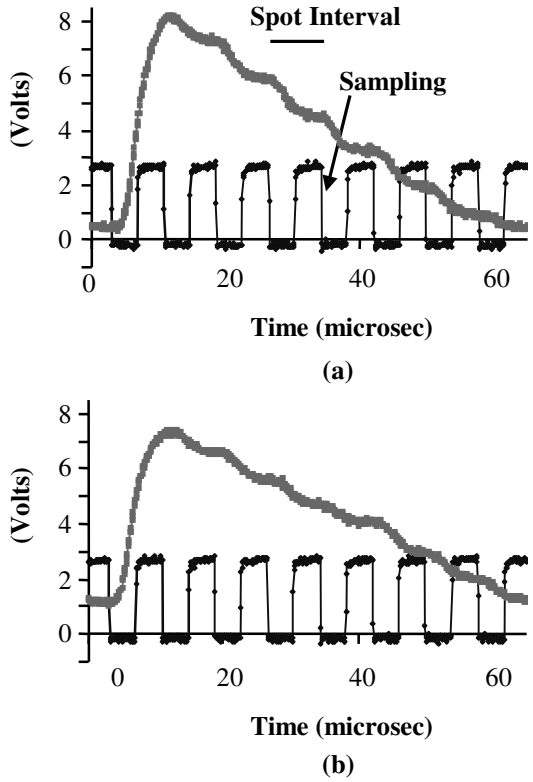
Instruments Code Composer Studio v1.2 and loaded onto the microprocessor board. The host program was written in Borland Builder C++ 5.0 and resides on the host computer. The programs interact through two "mailboxes" capable of exchanging single integer values and a "busmaster" transfer that can transmit data at speeds up to 20 megabyte per second bursts.

Although the host program and target program operate independently, they are synchronized by use of the mailboxes. First, the host program loads the target program, and tests are executed to ensure that initialization of communication is done correctly. Then, the target program generates 8-bit X and Y coordinates for each of the scanned laser spots according to the desired size and shape of the grid. Since the relationship between a Bragg cell's deflection angle and its driving signal is only approximately linear, the coordinates are linearized through a look-up table.

The target program then runs a normalization routine that attempts to correct for varying light intensities at different spots. Normalization is accomplished by taking scans of all the spots' fluorescence and finding the lowest fluorescence intensity. Laser power at each spot is adjusted using a method of successive approximation so that variance of brightness among spots is within approximately 10%.

Next, the target program initiates an interrupt linked to the M67's onboard precision (DDS) clock, and the main portion of the program enters a command handler loop that detects input from the host computer. In the interrupt, the X and Y coordinates and the normalized laser power level are sent to the digital I/O to control the intensity of the laser and the deflection of the beam to a grid pattern of spots. The digital I/O and external D/A converters Analog Devices AD558KD are used to control the Bragg cell deflectors because the rise-time of the A4D4's D/A converters is too long for our purposes. The interrupt occurs at a rate of 128 kHz or 1 cycle every 7.8 microseconds. The interrupt also controls the reading of data from the A/D channels connected to the photomultiplier tubes.

A ring buffer continually saves 2 seconds of data. When the command to digitize is received by the command handler from the host computer, the main loop of the target program triggers a flag, and the most recent 2 seconds of pre-triggered data are preserved. The collection of new data then begins when the laser beam moves to the first of the 128 spots; therefore, 1 millisecond is the longest delay to the collection of new data after the command to digitize is given. After the data is acquired, another flag is toggled allowing the transfer of information to the host computer. The pre-triggered data in the ring buffer is sorted into chronological order with the post-triggered data, and it is sent up to the host computer in a multiplexed stream through the busmaster transfer in 16 kilobyte bursts every 2 milliseconds. Transfer is slowed to this rate to allow the host computer time to collect the data and write it to RAM before new data arrives. After all the data is transferred, the host computer sorts it into individual channels for each PMT and writes it to hard disk. When transfer is done, the setup parameters are retained so that more data can be taken immediately.



3. Timing verification

Figure 1. Timing diagrams during scan of one row of laser spots. The square wave is the DDS clock and the other waveform is the a) green fluorescence or b) red fluorescence. In this test the fluorescence intensities of spots were made highly variable to reveal the settling time when the laser moved to each new spot. Sampling (downward deflection of DDS clock) occurred near the end of each spot interval, at which time the fluorescence signal was nearly settled.

Timing is a critical issue in the new laser scanner system. In order to verify that the correct data is being taken, it was first checked that sampling by the A/D converters is taking place at the proper time. To study this, we used the "DDS clock." This clock is linked to the sample and hold circuit of the A4D4 module and is

triggered by the initiation of the 128 kHz interrupt. To verify that sampling is taking place after the laser spot has moved and the fluorescence signal has settled, the photomultiplier tube output and DDS clock signals were passed to a digital oscilloscope. A sample of the oscilloscope readings for the non-normalized green fluorescence is shown in Figure 1a and for the non-normalized red fluorescence in Figure 1b. The DDS clock is the square wave with a 50% duty cycle. The downward deflection of the DDS clock waveform triggers the sample and hold circuit and initiates the interrupt. For all 128 spots, the trigger of the DDS clock was observed to take place after the fluorescent signal has settled.

Since delay exists from when the grid coordinates are sent out from the microprocessor's digital I/O to when the PMT outputs are sampled by the A/D converters, it is important to ensure that the correct spot location is being assigned to each sample. This was verified by blocking one spot of the grid with black electrical tape. Recordings were taken, and the blocked spot received a lower fluorescence signal than the unblocked spots. We found the interrupt set-up and equipment delays introduced a constant time shift for all samples. An adjustment was made in the analysis programs to compensate for the time shift.

Also to verify that each PMT signal was correctly digitized, a square wave signal was digitized and simultaneously observed on the oscilloscope. This revealed a 30% overshoot to a step response. The A/D converters of the A4D4 module were originally equipped with 6-pole anti-aliasing filters with 100 kHz passband, which produced overshoot. Since digitization could occur during the overshoot, we bypassed the filters with onboard jumpers in order to eliminate overshoot.

4. Animal tests

The laser scanner system was also tested with animal experiments. Isolated New Zealand White rabbit hearts were Langendorff-perfused as previously done [3]. The solution contained (mmol/L) NaCl 129, KCl 4.5, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 14, Na_2HPO_4 1, and glucose 11. It was bubbled with 95% 0_2/5% CO_2 at a pH of 7.3 to 7.4, an aortic pressure of 80 cm H_2O and temperature 37°C to 38°C. Borosilicate glass plates were positioned on the recording region of the heart to provide a flat recording surface. In these particular experiments, a thin layer of indium tin oxide was present on the surface of the plates for studies of electrical stimulation. Recordings were taken on the ventricular epicardium.

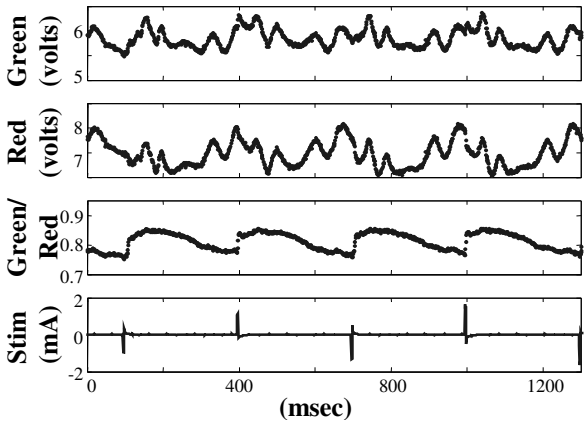


Figure 2. Signals collected at one of the 128 spots scanned. The x-axis represents time in milliseconds. The first two graphs are of the green and red fluorescence, respectively. The third graph is the ratio of these green and red signals. The fourth graph is the stimulation current with alternating polarity delivered to the heart with a bipolar electrode on the epicardium.

The hearts were stained with di-4-ANEPPS (Molecular Probes, Inc.) added to the perfusate. An argon laser emitting 488 nm light scanned a grid of 8 x 16 spots in a 0.6 cm x 1.2 cm region to excite the di-4-ANEPPS. Fluorescence following the transmembrane voltage changes was collected by two photomultiplier tubes oriented 90° to one another. Light was separated with a beam splitter, a 590 nm long-pass filter for red light and a 510-570 nm band-pass filter for green light. Also, one A/D channel was used to monitor laser intensity level, and another recorded the stimulation current delivered to the heart. Four to twelve seconds of data were recorded in each trial, and the data was later demultiplexed using MatLAB 6.1 to obtain individual recordings for each spot and light color.

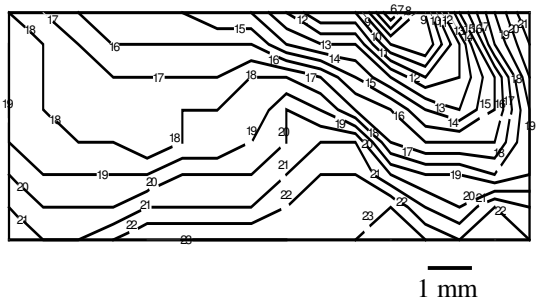


Figure 3. Action potential propagation map for the 128 spot grid. Isochrone contours representing times of the phase-zero depolarizations of the cells at the laser spots are shown in 1 millisecond increments. The action potential first appears in the upper right and then propagates in the entire scanned region.

An example of the signals collected is shown in figure 2. An action potential is indicated by a change in the fluorescent signal when the cells depolarize preceding the motion artifact [1]. This is seen as an upward deflection in the green signal and a downward deflection in the red signal. Additional deflections also occur in the green and red signals due to heart motion artifacts. Emission ratiometry, dividing the green signal by the red signal, reduces motion artifacts [4]. The action potential can be clearly seen in the ratiometric signal (third graph).

Propagation in the entire scanned region for the first action potential in figure 2 is shown in figure 3. The activation time was detected for each spot by finding the time of maximum upward slope in the ratiometric signal. These times were then plotted on a contour map. The figure shows the action potential spreading in the grid from the upper right region.

5. Conclusion

The laser scanner system can collect fluorescence from 128 spots with multiple photomultiplier tubes every millisecond. The collection of more than one band of wavelengths of fluorescence allows the use of emission ratiometry to reduce motion artifacts. This aids in action potential detection and observation of propagation through the 2D area of the grid. The use of the microprocessor allows considerable flexibility in future applications. The shape of the grid can be readily changed. Fewer grid points can be used to allow for a faster scanning rate. Also, additional PMTs can be added to measure different wavelength bands, which may be useful for coloaded dyes or new dyes with different emission characteristics.

Acknowledgements

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