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A STIMULUS TIMING DEVICE FOR CAPTURING FAST PHYSIOLOGIC EVENTS BY QUICK-FREEZING

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Abstract

A timing device was designed that, in conjunction with an impact type of quick-freezing apparatus and an externally-triggerable stimulus generator, allows the application of an electrical stimulus to a muscle preparation at a selected time interval before quick-freezing and the measurement of the interval with sub-millisecond precision. This is needed for stopping fast physiological events in calcium release and excitation-contraction coupling and allows studying the morphological parameters (by freeze-fracture and freeze-substitution) and elemental distributions (by x-ray microanalysis) as a function of time after stimulation. The device should be adaptable for use with most equipment designed for quick-freezing electrically excitable tissue by impact on a cold solid surface.

KEYWORDS: Quick-freezing, cryopreparation, cryofixation, skeletal muscle, excitation-contraction coupling, instrumentation, timing, methods, x-ray microanalysis

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Introduction

Studies of the in vivo structure and topochemistry of cells and tissues demand that necessary preparatory procedures preserve the in vivo state with high fidelity. Quick-freezing is a widely used procedure that serves this purpose in a unique way [2,7,12,14,16,18,19,21]. Since, in contrast to chemical fixation (which takes place on a time-scale of seconds and minutes), the process of quick-freezing is very fast, at least for narrow ($\approx$ 1-3 μm) superficial regions of interest close to the point of first impact [1,3,4,9,10,14], it is possible to arrest and study fast physiological events [2,5,6,13,15,16,20,21]. For example, quick-freezing muscle cells following excitation allows preserving and studying both the geometry of structure and the elemental distributions that existed in the tissue at the time of freezing at very high temporal and spatial resolution [8,11,14]. By comparing freeze-fracture images with those resulting from freeze-substitution following quick-freezing, and by judging the state of cryopreservation with a set of rigorous criteria, we have demonstrated excellent structural agreement between the results of these two independent procedures [2,14,16], one of which offers images of the frozen-hydrated state of the muscle cells. In addition to preparing thin sections of embedded freeze-substituted and freeze-fractured or freeze-etched replicas of muscle fiber segments, which can be analysed by high resolution EM, we have obtained freeze-dried cryosections from the same cell, allowing quantitation of elemental concentrations, especially of Ca, at known time intervals between electrical stimulation and quick-freezing [e.g. 13,14]. The temporal coincidence of elemental displacements, e.g. of Ca, and morphological events can then be correlated at the ultrastructural level [6,14, 17,20,21], possibly leading to new

insights into the calcium release mechanism.

We have designed a timing device (hereinafter referred to as "device") that gives accurate and reasonably reproducible stimulus-to-freezing time intervals (SFI) and which can be used with a quick-freezing apparatus that impacts the specimen onto a cold solid surface, similar to the Heuser design [7]. We measure the SFI as the time interval between the application of the electrical stimulus to the preparation and the time the preparation impacts the cold copper block. We have used the device with both the Polaron Slammer (Polaron Instruments, Cambridge, MA, USA) and the Cryopress (Med-Vac, Inc., St. Louis, MO, USA).

The requirements that this timing device was designed to satisfy were: (1) provide accuracy and resolution of better than 1 ms, (2) provide good reproducibility of the SFI, and (3) interface easily with the existing quick-freezing equipment and stimulus generator.

Circuit Design

General Description of Operation.

The specimen (in our case an isolated, single intact frog skeletal muscle fiber) is mounted on a specimen holder on a gelatin base deposited over an aluminum disk (see [14] for details). The specimen holder is inverted and mounted at the bottom of the plunger of the quick-freezing apparatus. The plunger is released by a solenoid and falls by gravity, guided by bearings; the specimen is quick-frozen at the end of the plunger travel when it hits the polished surface of a liquid-helium cooled copper block. A more detailed description of the Cryopress is given in Heuser et al. [7] and Padron et al. [15].

The device was designed to allow the user to set the SFI on a 10-turn dial according to a calibration chart. The device sends a trigger pulse to the stimulus generator and a pulse to the plunger release solenoid timed to provide the desired SFI. A separate circuit measures the actual time between the application of the stimulus and the impact of the specimen with the copper block.

Timing ranges.

There are two timing modes: Long (Figure 1(a)), in which the stimulus generator (Grass Instrument Co., Quincy, MA, USA, model S48) is triggered before the plunger is released in order to allow SFIs greater than the time taken for the plunger to drop; and Short (Figure 1(b)), in which the plunger is released before the stimulus is applied. In implementing the circuit, we have divided the Short mode into two ranges, Short and Medium which cover SFIs of about 0-12 and 0-150 ms; Long covers intervals longer than 100 ms (time selected depends on the time it takes the plunger to drop for a particular machine).

Circuit Description.

The circuit was designed around the inexpensive 555 timer chip. The device interfaces with the quick-freezing machine's main controller through two wires that connect to the controller's plunger "drop" push-button switch, allowing the device to control the time at which the plunger is released while the controller retains all auxiliary functions (opening the shutter and activating the anti-bounce magnet).

The circuit was built on commonly available circuit board. Large capacitors in the timing circuit were tantalum type. Outputs to the interval timer and the stimulus generator were through coaxial connectors, and to the main controller and the end-point circuit through appropriate pin connectors. We used a Hall-Effect magnetically-activated switch in order to avoid bounce that may be introduced by a reed switch; it is also somewhat simpler to implement than an optical (emitter-detector-shutter type) switch. The circuit is powered by a regulated bipolar 9-volt supply. A five-pole three-position wafer switch selects the timing range desired. The switching details are shown in figure 2.

Long interval mode.

The push-button switch triggers two 555 timers, **A** and **C** (Figure 1(a)) simultaneously. **A** outputs a pulse of fixed duration (about 1 s; this determines the maximum SFI that can be used). The falling edge of the pulse triggers **B** which in turn generates a pulse that turns on a vertical-groove metal oxide semiconductor (VMOS) field effect transistor switch (VN67AF) connected in parallel with the plunger release switch on the main controller, activating the release solenoid. **C** gives a pulse whose width determines the interval between the time the push button is pressed and the time the stimulus is applied and, therefore, the SFI. The falling edge of the output pulse of **C** triggers another 555 timer, **D**, which outputs a narrow pulse that triggers the stimulator, which, in turn, applies the stimulus to the preparation.

Short interval mode.

In this mode (see Figure 1(b)), the push-button switch triggers timer **B** directly which drives the VMOS switch causing the plunger to be released. Once the plunger starts on its downward travel, a magnet attached to it activates

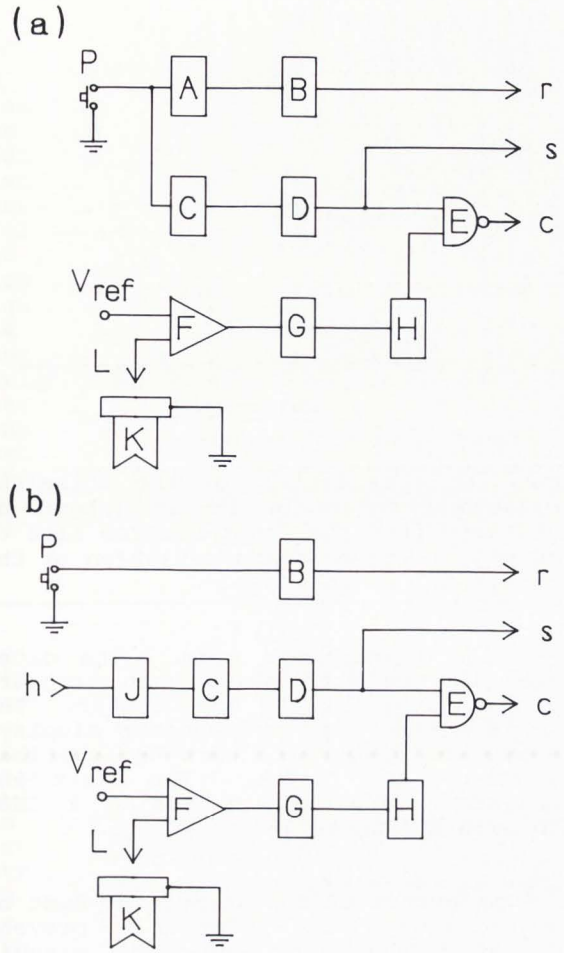

Figure 1. Block diagram. **A, B, C, D, G, J** are 555 timer integrated circuits; **E** is a NAND gate, **H** is a monostable multivibrator. **r** goes to the VMOS switch controlling the release solenoid; **s** goes to the stimulator trigger input; **c** goes to the digital counter input; **h** is the Hall-effect sensor output. **D** triggers the stimulus generator and simultaneously starts the digital counter, operated in period mode, at the end of a delay determined by timer **C** (see text). A separate circuit, consisting of comparator **F**, timer **G** and "one-shot" **H** detects impact of the specimen (via electrode **L**) with the cold copper block **K** and stops the digital counter, which now indicates the SFI. **E** combines the start and stop pulses to the digital counter. **(a)** SFI longer than 100 ms: the push-button switch **P** starts a timing cycle that generates the stimulus trigger pulse before the plunger is released (see text). **(b)** SFI shorter than 150 ms: switch **P** releases the plunger before the stimulus is applied, the timing cycle being started by the output of the Hall-effect sensor **h** after the plunger starts to drop.

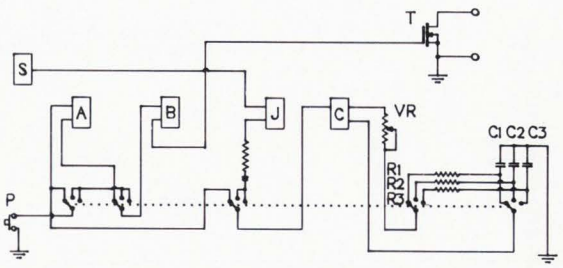

Figure 2. Switching diagram. The Hall-effect digital switch is mounted on the backplate of the Cryopress facing a magnet that is mounted on the plunger rod. **A, B, J, C** are 555 timer chips (see Fig. 1 for block diagram). The Hall-effect switch triggers **J** which starts the timing sequence in the Medium and Short modes. A 5-pole 3-position switch is used to select the timing ranges (positions 1, 2, and 3 correspond to timing ranges Long (> 100 ms), Medium (0 - 150 ms) and Short (0 - 12 ms). VR is a 10-turn potentiometer fitted with a 10-turn dial used to set the stimulus-to-freezing interval determined by **C**. R1, C1, R2,C2, and R3,C3 are resistor-capacitor pairs that determined the three timing ranges. The VMOS power transistor (VN67AF) is used as the plunger-release switch and is connected across the manual plunger release switch on the Cryopress control box.

a Hall-effect digital switch fixed to the backplate of the Cryopress. The output from the switch triggers a 555 timer, **J**, which outputs a short pulse (pulse width about 1 μs, which adds negligible delay) that triggers **C**. The pulse width of **C** determines the time at which the stimulus is applied and triggers **D** which in turn triggers the stimulator as described above. This arrangement, whereby the timing cycle is started by the movement of the plunger, improves the reproducibility of the timing by removing from the timing sequence the variable delay introduced by the plunger release solenoid.

End-point circuit.

This circuit produces a pulse when the specimen impacts the grounded copper block. The aluminum disk under the specimen on the specimen holder is connected to one input of a comparator **F** and to the -9 volt power supply through a 4.7 megohm resistor (current is limited to less than 5 μA). The specimen holder assembly is isolated from ground. When the specimen touches the copper block, the output of the comparator changes polarity and causes the gate of the

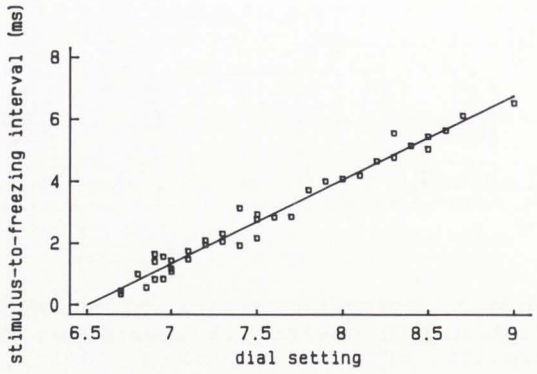

Figure 3. Stimulus-to-freezing intervals obtained in the short interval mode at various settings of the dial (VR in Fig. 2). Least squares fit is shown (r^2=0.97). Device used with the Med-Vac Cryopress.

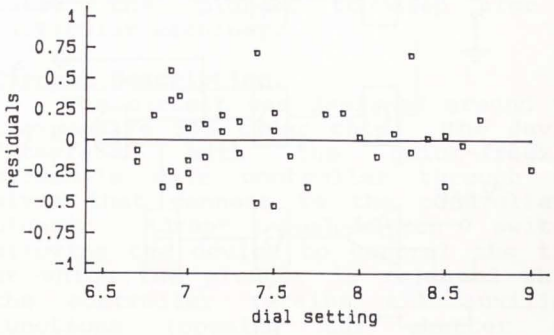

Figure 4. Variability of the stimulus-to-freezing intervals in ms (shown as deviations from the least-squares line of Figure 3). The standard deviation of the error is 0.3 ms (n = 38).

frequency counter/timer to be closed (it is necessary to ensure that grounding the copper block does not interfere with the thermocouple circuit should the thermocouple in the cryoblock come into electrical contact with the copper block: it may be necessary to isolate the thermocouple circuit from ground).

In order to prevent spurious triggering of the gate of the digital timer immediately after the specimen hits the copper block (and thus lose the displayed time, or, worse, get an erroneous display), a 555 timer **G** was added and arranged to give a long pulse which inactivates the circuit for the duration of this pulse (a few seconds). The leading edge of the pulse output from G triggers the complementary MOS (CMOS) "one-shot" **H** that closes the digital timer gate.

Guard circuit. Because the end-point circuit was designed to have high-resistance and low-current, it would be possible for current leakage e.g., when the metal bell housing of the specimen holder slams into the magnet assembly, to give a false reading (but reasonable in magnitude) should the true end point fail to register for some reason. In order to obviate this possibility, a 9-volt battery was connected (through a current-limiting resistor) between the magnet assembly and ground. The magnet housing-copper block assembly on the Cryopress made this easy as it is insulated electrically from the frame of the machine through its nylon shock mounting.

Measuring stimulus-to-freezing interval.

In both the Long and the Short modes the SFI interval is measured using an inexpensive digital frequency counter (Heath/Schlumberger Model SM-4100), used in period measurement mode. The gate, opened by the same pulse that triggers the stimulator, starts the counter. The gate is closed, and the counter displays the measured interval, when the specimen hits the copper block. The start and stop pulses are combined using a CMOS NAND gate **E** (Figure 1).

Stimulus isolation.

The output of the stimulator must be isolated from ground in order to prevent coupling between the end-point circuit and the stimulus circuit which might cause spurious triggering of the end-point pulse. If the output of the stimulator is not so isolated, a stimulus isolating unit with low capacitance between output and ground (e.g. Grass Instruments type SIU5, which we currently use) or a suitable optical isolating unit should be used.

Plunger alignment.

Since the plunger drop Hall-effect sensor can be activated by simply rotating the plunger rod on which the magnet is mounted, and in order to improve reproducibility of timing by having the plunger always start with the magnet in the same position relative to the sensor, a Lucite plate, mounted horizontally on the plunger just above the specimen holder housing, automatically aligns the plunger when raised. Set screws provide adjustment for the clearance between the back of the Lucite plate and the backplate of the Cryopress. The Lucite plate also serves as a convenient terminal block for the stimulus and end-point circuit connections.

Results

The variability in the SFI obtained is largely attributable to the quick-freezing apparatus and the positioning of the specimen on the specimen holder; the additional variability contributed by the electronics is negligible. Figure 3 shows the SFIs obtained as a function of dial setting (Short Mode) using the Med-Vac Cryopress; the least-squares regression line is shown ($n = 38$; $r^2=0.97$); Figure 4 shows the error (deviations from the regression line) plotted as a function of dial setting. The standard deviation of the error was 0.3 ms in the Short and Medium interval modes. In the Long interval mode, we obtained a somewhat higher standard deviation, 0.7 ms ($n=16$), which is likely to be due to the additional variability introduced by the release solenoid.

Discussion

The actual freezing rate leading to the immobilization of intracellular components within the cells under study depends on a number of factors, such as the nature of the coolant, i.e. its heat capacity and conductivity, and its temperature [1,3,4,10,11,14,18]; it is subject to variations in the thickness of the water jacket covering the specimen just before freezing. The actual intracellular temperature gradients and the degree of cryoprotection rendered by the cell substance itself and, thus, precisely when immobilization of cell constituents occurs remain unmeasurable. The best estimates are that within the first 1-3 μm of the point of first impact, immobilization takes place within a few fractions of a millisecond. In isolated skeletal muscle cells a continuous gradient of cryodamage with almost imperceptible transition from the point of first impact to more distant portions of the cells has been established which may serve as a rough guide to freezing rates [14,16]: only freezing rates at, or close to, those required for so-called vitrification will cause tissue to remain free of cryoartifacts as determined by using electron microscopy at very high spatial resolution.

In the current device, we use the time of impact of the preparation with the cold copper block as the time of freezing. One can easily adapt the device to any other time-of-freezing criterion that can be made to provide an electrical signal (e.g., changes in capacitance [6], the output of an imbedded thermocouple, etc.)

Although the accuracy of the measured SFI is not affected by variation in the time it takes the plunger to fall, the reproducibility is. Some factors that, in our experience, can affect the reproducibility of timing are:

1. Variability in the operation of the release solenoid (affects the "Long" mode only). The release catch may be polished to reduce the friction [15].
2. The plunger might bind on its downward travel: The plunger rod should be polished, clean and dry. Bearings should be free, must be adjusted to prevent even the slightest binding of the plunger, and should not rotate or should just barely rotate at the end of the plunger's travel. The machine should be leveled such that the plunger rod is truly vertical.
3. Wires connected to the plunger may interfere with its movement: Wires should be of stranded, flexible construction and be routed such that they will provide minimum interference.
4. If the liquid helium tank is not depressurized early enough before releasing the plunger, this would create a variable force on the descending plunger that can retard it as much as a few milliseconds and which, at first sight, is hard to trace. We have found that it was necessary to depressurize the tank three to five seconds before dropping the plunger to get consistent time intervals. The quality of the tissue preservation does not seem to be adversely affected by this delay.
5. The dropping distance (between the specimen and the top surface of the copper block) may vary resulting in changes in the SFI. For example, the thickness of the gelatin "cushion" on which the specimen is mounted may vary from specimen to specimen; or the anti-bounce spring-loaded specimen holder may bind in other than its fully extended position at the time of dropping.

In this design we have attempted to attain good accuracy and reproducibility of the SFI. The accuracy is determined by the accuracy with which the "instant" of quick-freezing can be determined (the time of application of the electrical stimulus is accurately known, and the time of stimulation is known to within the width of the stimulus pulse-- the pulse should be shorter than the desired accuracy of measurement). Since the SFI is measured, its accuracy does not depend on the assumption of frictionless free-fall of the plunger. The device is simple to design and construct, can be adapted to different designs based on the Van Harreveld-Heuser quick-freezing apparatus [7,20], and requires minimum modification of the apparatus controller.

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Discussion with Reviewers

C. Franzini-Armstrong: This device is designed with the aim of obtaining time resolution of one msec. In this case,

the thickness of the gelatin layer under the muscle fiber must be critical. I am curious to know whether the authors carefully determine the position of the muscle fiber relative to the aluminum disc, or whether they simply determine impact time, and then use the fibers that happen to be frozen at the appropriate interval.
Authors: The plunger on our slamming machine falls a distance of about 20 cm; assuming a free fall, a change of 1 mm in the position of the fiber relative to the aluminum disk will change the stimulus-to-freezing interval by about 0.5 ms. We try to keep the distance as constant as possible when we mount the fiber. We agree that measuring this distance and taking it into account when setting the stimulus-to-freezing time should result in improved reproducibility. We have been interested in a range of stimulus-to-freeezing intervals rather than one specific time, so the variablity we get around the set time is not a great disadvantage, as long as we measure the time with the desired accuracy.

K.P. Ryan: Could the device be adapted for use with plunge-freezing?
Authors: The difficulty in applying the device for use with plunge-freezing lies in the absence of a discrete, fixed point (analogous to the surface of the cold copper block) that could be used to define the time of freezing. Heuser's capacitance method [6], with appropriate calibration, could be used to trigger comparator F (Figure 1). Alternatively, perhaps one could use a very fine thermocouple or thermistor to trigger the comparator, the appropriate trigger point being perhaps initially determined by an auxiliary calibration using the capacitance method.

K.P. Ryan: Discussion of the potential problems is very helpful. Could the authors explain further the variable force deriving from the LHe tank?
Authors: We think that the increased relative velocity between the falling plunger/specimen holder and the rising gas (when the tank is not depressurized well before dropping the plunger) increases the frictional resistance sufficiently to cause an increase of one or two ms in the time required for the plunger to drop.

K. Zierold: Have you found differences in the quality of cryofixation by cooling the metal block with liquid nitrogen in comparison to liquid helium?
Authors: We have not tried liquid nitrogen to cool the metal block.