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Hogan, P.M. and Besch, S.R.

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We have spent the past year designing, building and testing the components of a microfluorimeter system for measuring cytosolic calcium in single cardiac cells at high hydrostatic pressure. The central elament of the system is a 75 microliter cell chamber fitted with windows and placed in the optical axis of a Zeiss inverted microscope. An optical network has been devised to record simultaneously the fluorescent emission from FURA-II, a calcium reporter dye, and sarcomere spacing from differential interference contrast imaging. To achieve the required microsecond time resolution and sub-micrometer space resolution we have integrated optical, mechanical and electrical components using an 80386/80387 central processor with custom interface hardware and software. Details of these technical developments are provided in our annual report.

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PRINCIPAL INVESTIGATOR: Perry M. Hogan

COINVESTIGATOR: Stephen R. Besch

CONTRACTOR: Research Foundation of State University of New York

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INTRODUCTION

We have spent the past year designing and building a miniature compression chamber and a computer based microfluorimeter system for measuring intracellular calcium in physiologically viable cardiac cells. This report is divided into the following sections that describe the relevant details of our technical progress.

Integrated system design

High pressure cell chamber

Plumbing system for pressure chamber

Computer hardware interface

Computer software operating system

Integrated system design: Figure 1 is a schematic diagram showing the essential elements of the microfluorimeter system. A cardiac muscle cell is isolated and placed in the 75 microliter cell chamber (CC) that is located over the objective lens (OL) of the Zeiss inverted microscope. Details of the cell chamber are provide below. Windows on the top and bottom of the chamber permit illumination of the cell and excitation of the calcium sensitive fluorescent dye (FURA-II) used to measure intracellular calcium. The ratio method for measuring calcium requires that two excitation wave lengths (340 & 380 nm) be applied alternately to the cell containing FURA, while emission photons are gathered at

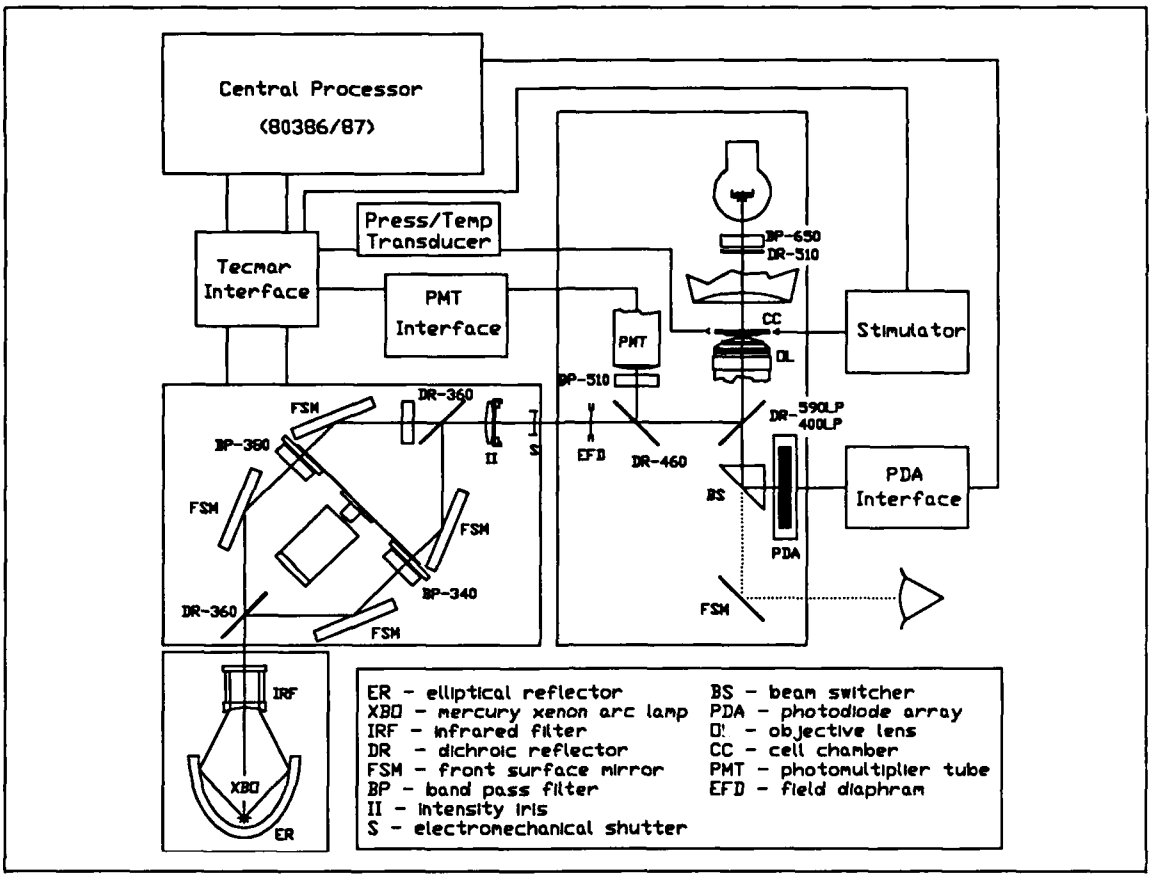

Figure 1

510 nm. The ratio of emission intensities for each pair of excitation exposures yields a measure of intracellular calcium. Broad band light from a mercury xenon arc lamp (XBO) is passed through an infrared filter (IRF) and then through a system of dichroic reflectors (DR) and band pass filters (BP) of appropriate characteristics to isolate the excitation light and sequence its presentation to the epifluorescent input port of the microscope. Sequencing is accomplished using a spinning 30 sector aperture wheel and adjustable masking slits. Details of this technique were given in last years annual report. The excitation light is then guided by dichroic reflectors to the objective len of the microscope where it is focused on the specimen.

Fluorescent emission photons are collected by the objective lens and guided by dichroic reflectors to the photomultiplier tube where they are counted and stored in the computer. Simultaneous with these measurements, differential interference contrast (DIC) images are gathered to assess the contraction state of the cell. To prevent contamination of the fluorescent measurements a bandpass filter is used to select a DIC illumination wavelength of 650 nm. The DIC image is focused on a 1024 element, linear photodiode array (PDA). Images are recorded using a custom designed PDA-computer interface for throughputs of up to 5 megahertz - for details

see below. The spatial resoluton of the PDA image is a quarter of a micron.

In addition to the PDA interface, we have incorporated a TECMAR multifunction laboratory interface to record PMT data and to continuously monitor the hydrostatic pressure and temperture of the cell chamber. In addition, the TECMAR interface is used to set parameters for the excitation light subsystem, synchronize the application of the stimulus and coordinate the acquisition of data. The entire process is under the control of custom software to be described in a later section.

High pressure cell chamber: This section is intended to expand upon the preliminary information provided in last year's report. Since that time, the single cell pressure chamber has been completed and put into service. A cut-away view of the chamber is shown in figure 2.

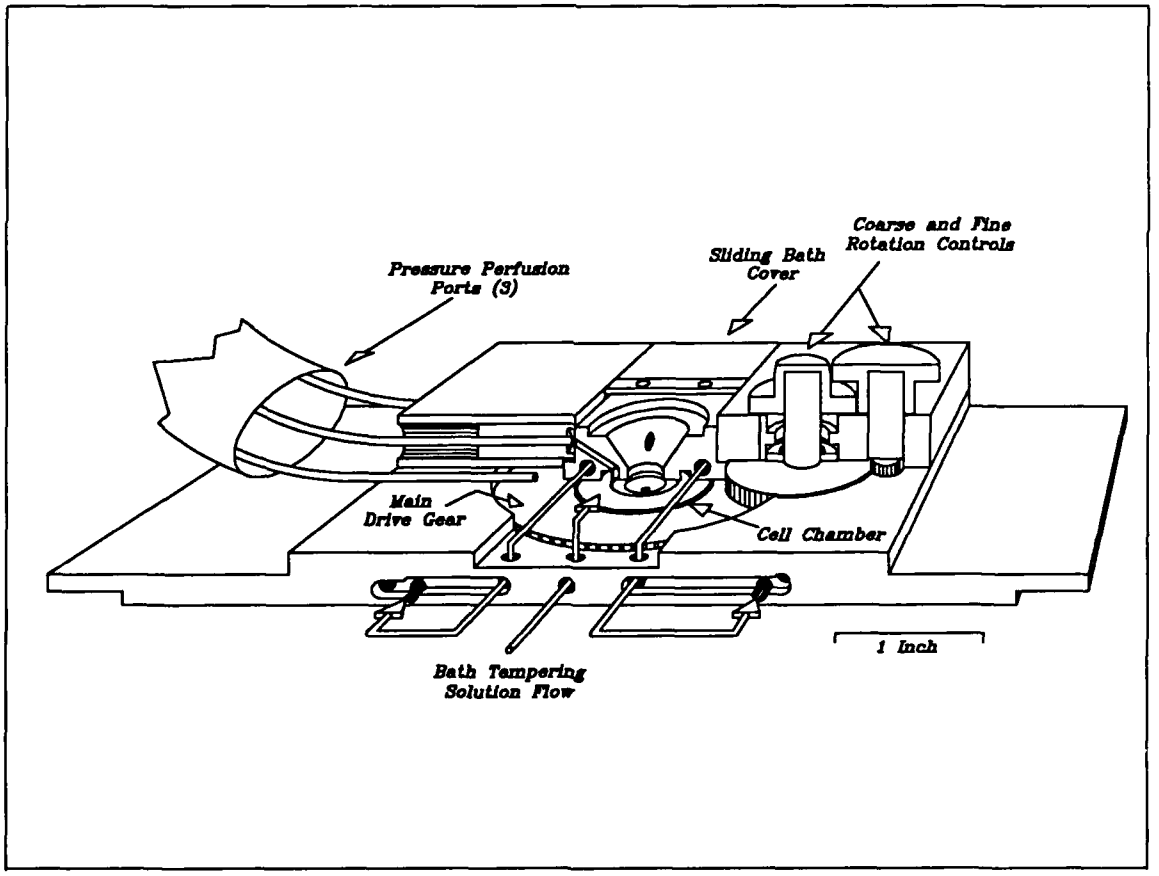

Figure 2

The central component of the device is the cell chamber. Fabricated from aluminum and anodized, it is a small (0.950" O.D., 0.075" thick) button with a

complex conical well bored through it. The narrow end of the conical well is 1 mm in diameter and has a 0.17 mm quartz cover slip glued over it to serve as the pressure window. The cardiac cell under study is attached to the inside surface of this window. The opposite side of the button provides a surface against which a neoprene O-ring forms a pressure seal when the sliding chamber cover is in place.

The cell chamber is in turn mounted in a cavity at the center of rotation of the main drive gear. This is a stainless steel gear whose function is to allow the rotation of a cell in the chamber into proper alignment with the excitation light slit and photodiode array while the cell chamber is pressurized. Two stages of gear reduction are provided to facilitate the precise control of rotation required at high magnification and at high pressure. The main drive gear rotates in a large diameter angular contact ball bearing to provide support against the large thrust load when the chamber is fully pressurized.

The anodized aluminum base of the pressure chamber mounts directly to the X-Y stage of our Zeiss inverted microscope to provide complete freedom of movement of the pressure chamber in the optical field of the microscope. The main drive gear and the secondary reduction gears fit into cavities bored into this base component. A slot is milled into the center of the base to act as a guide for the sliding chamber cover, which is held in place by a pair of retainer blocks mounted on either side of this slot. The left hand member of this pair provides access ports to the sliding chamber cover.

A considerable portion of the engineering work on this device went into the design of the sliding bath cover. It allows opening the pressure chamber and changing the cell chamber button in only a few seconds, while providing a back side pressure window for the transillumination of the cell chamber for the microscopic observation of the cells in the chamber. The basic design is a rectangular block with ears which slide under the retaining blocks mounted to the base plate. In the center of the block is a conical hole which matches the numerical aperture of the microscope condenser lens. The bottom side is closed with a 12 mm diameter quartz pressure window, while the top side is sealed with a piece of 1 mm thick microscope slide. During operation, the space between these 2 windows is filled with the heating/cooling solution which flows through the cover. A neoprene O-ring is located around the quartz pressure window to seal off the cell chamber when the lid is in place.

Three 1 mm holes are concentrically located in the space between the O-ring pressure seal and the quartz pressure window. These communicate with holes located in a line along the left side face of the cover (see figure 2) and provide hydraulic access to the cell chamber, allowing pressurization, monitoring and perfusion of the chamber. These holes in turn mate with the O-ring sealed access ports correspondingly placed in the left side cover retaining block.

The last feature of this chamber is the provision made for temperature control. Both the base and the sliding chamber cover contain ports which communicate in such a way as to provide a circulation pathway for heating/cooling water. A water inlet directs flow into the chamber cover via an O-ring seal at the front edge of the cover guide slot. Water flows directly into the conical well formed in the cover for trans-illumination light. From here, water flows to the rear of the cover where it branches and flows back to the front through holes located at either side. These holes communicate with the base via the remaining 2 O-ring sealed ports at the front edge of the guide slot. From here, water is routed through holes to the rear of the base, where it is collected at an outlet port. Thermally tempering the entire chamber structure eliminates thermal gradients away from the cell chamber itself, leaving only the objective lens as a potential heat sink. Thermal tempering of the objective lens is also possible and relatively straightforward.

We have been using this chamber routinely at pressures of 70 ATA with no visible degradation of the window attachment to the pressure vessel. Experiments in our laboratory have shown that pressures in excess of 200 ATA are obtainable with this system, although some degradation of the cover slip attachment occurs. This was described in last years report.

Plumbing system for pressure chamber: Figure 3 illustrates the plumbing network for the high pressure cell chamber. The chamber appears as an expanded space in a perfusion line driven directly by a constant flow HPLC pump through an injection valve and into an adjustable pressure load to provide continuous flow independent of pressure. The addition of the injection valve to the circuit allows the precise introduction of materials into the perfusion stream

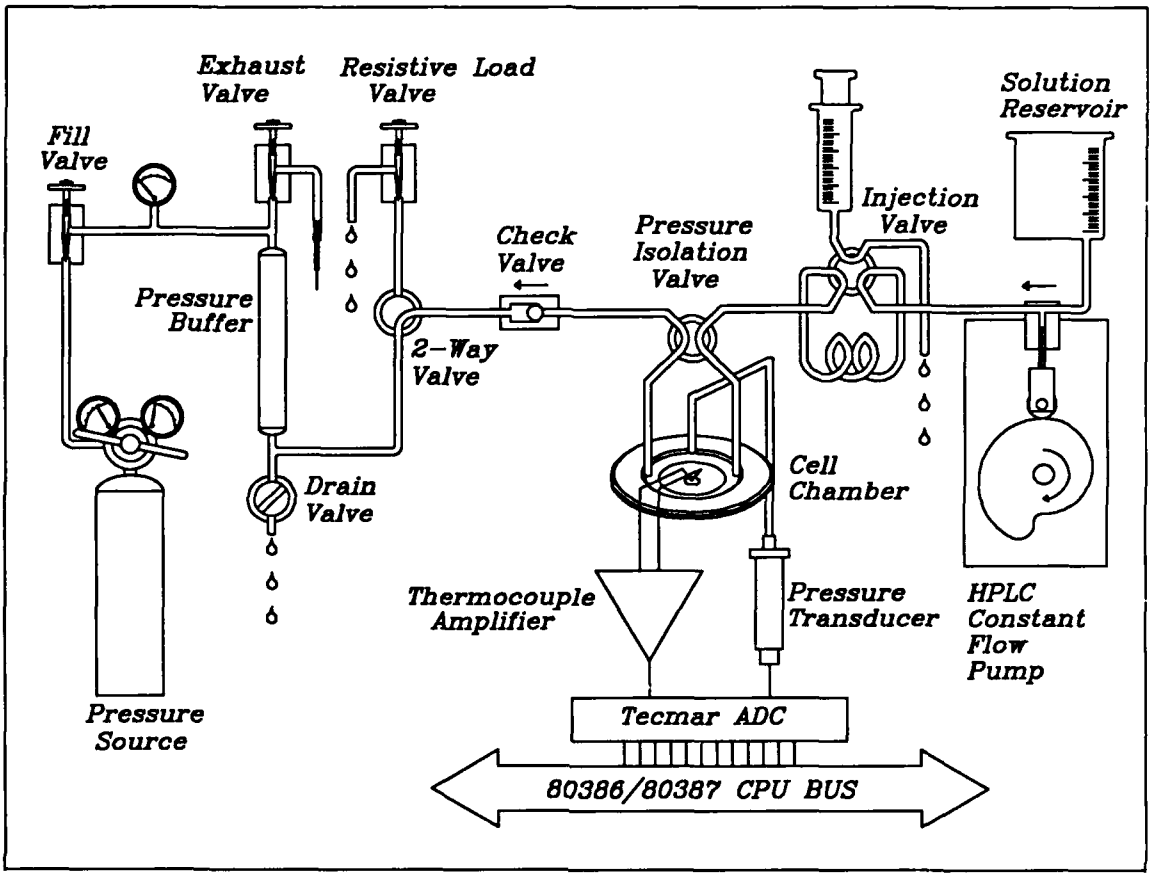

Figure 3

at pressure. The absence of any gas volume over the chamber allows rapid attainment of a target pressure without the adiabatic thermal effects associated with gas phase compression over liquid. Furthermore, the direct hydraulic compression of the perfusing solution eliminates the absorption of compression gas into the perfusate.

There are 2 distinct modes of pressure loading. The first is a simple needle type metering valve which provides a resistive load whose purpose is to provide a positive visual confirmation of flow at pressure. With flow rates below 200 ul/min., this type of load suffers from significant pressure fluctuations even with the best HPLC pumps. With the needle wide open, this pathway can provide a convenient 1 ATA exhaust port.

The second type of pressure load is a 2 liter buffer bottle into which the perfusate is pumped. The bottle can be charged to any pressure within the operating range of the cell chamber. As long as the bottle is kept relatively empty of fluid, the perfusate addition will have an insignificant effect on pressure. A drain valve allows for the periodic emptying of liquid from the bottle. This loading method provides a constant chamber pressure in spite of small flow fluctuations in the HPLC pump

and is the preferred method for most experimental conditions.

The perfusion lines to the chamber are routed through a crossover type valve to provide a means of pressure isolation of the cell chamber. The valve is a "make before break" type of valve so the pump never pumps against an infinite load during valve transitions. This valve allows rapid isolation of the chamber at elevated pressure (and zero flow). During pressure isolation, the buffer bottle can be precharged to a new pressure while the bath is held at the original pressure. Switching the chamber out of isolation after the bottle transition provides a nearly instantaneous step in pressure. The pressure isolation valve also has the obvious use of isolating the bath during depressurization for the purpose of changing the chamber button.

Plumbing connections to the cell pressure chamber are made with flexible narrow bore PEEK tubing to permit unlimited movement of the chamber structure. A significant, and potentially dangerous, amount of energy is stored in the buffer bottle when operating at high pressure. To protect the microscope (and the operator), a check valve is located at the buffer bottle to isolate this energy source from the PEEK tubing and the cell pressure chamber in the event of a tubing failure or a window rupture.

Figure 3 also illustrates the pressure/temperature measuring capability of the system. Pressure is monitored directly at the cell chamber by a solid state strain gauge which is connected to the third chamber access port. The absence of flow in this connection guarantees that there will be no pressure error due to flow resistance in the narrow bore tubing. This can be a significant error in tubing with a 0.005" ID, even at low flows. Temperature is measured using a small diameter (25 um) thermocouple installed directly in the open aperture of the chamber button. Both temperature and pressure transducer outputs are sampled by the TECMAR A/D converter and recorded with the corresponding experimental data.

Computer hardware interface: The computer hardware interface is designed around a Tecmar Lab Master DMA-100 multifunction board. Figure 4 shows a schematic representation of the deployment of board functions among the operators of the system. Digital output ports are used to select/toggle elements of the excitation light subsystem. These include power status, iris aperture, masking slit width, and shutter status. Digital-to-analog converters (DAC's) are used to control aperture motor speed and stimulus intensity. Digital input ports are used to monitor the status of the right and left apertures of the spinning sector wheel. Timing pulses indicating the open/close status of these apertures are used to synchronize the gathering of fluorescent emission photons and their storage

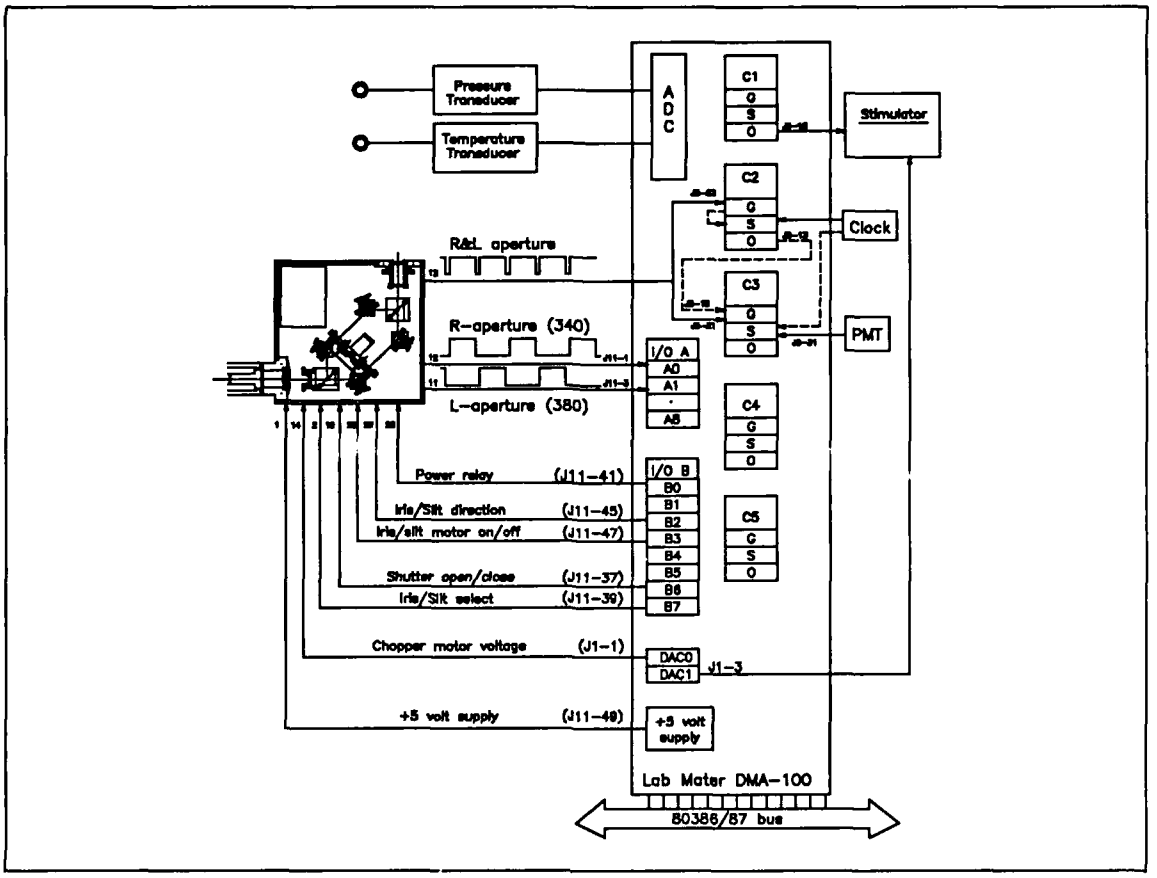

Figure 4

in the appropriate collection bins. Two 16 bit counter registers are used to simultaneously time each aperture-open-interval and count the number of photons emitted during the interval. A third counter is used to determine the frequency and duration of the stimulus pulse. Finally, two analog-to-digital converter (ADC) are used to record temperture and pressure in the cell chamber.

In addition to the Tecmar interface we have developed a custom interface to input differential interference contrast (DIC) images of the contracting cardiac cell (figure 5). We use a change of sarcomere spacing as a physiological correlate to an intracellular calcium change. The magnified DIC image is projected onto a 1024 element linear photodiode array (EG&G Reticon) whose video output is digitized using an 8-bit flash converter (Analog Devices AD9048) and stored in computer memory for later processing. Although our present level of design limits us to data rates of 256,000 pixels/sec, with further development, data rates as fast as 5 million pixels per second are possible.

Implementation of this methodology required the development of electronics for the flash converter, and a suitably designed memory array. Both of these items were designed and built in house. The flash converter interfaces with an EG&G

development board and includes a 1024 byte FIFO, control logic and transmission line drivers to transfer the

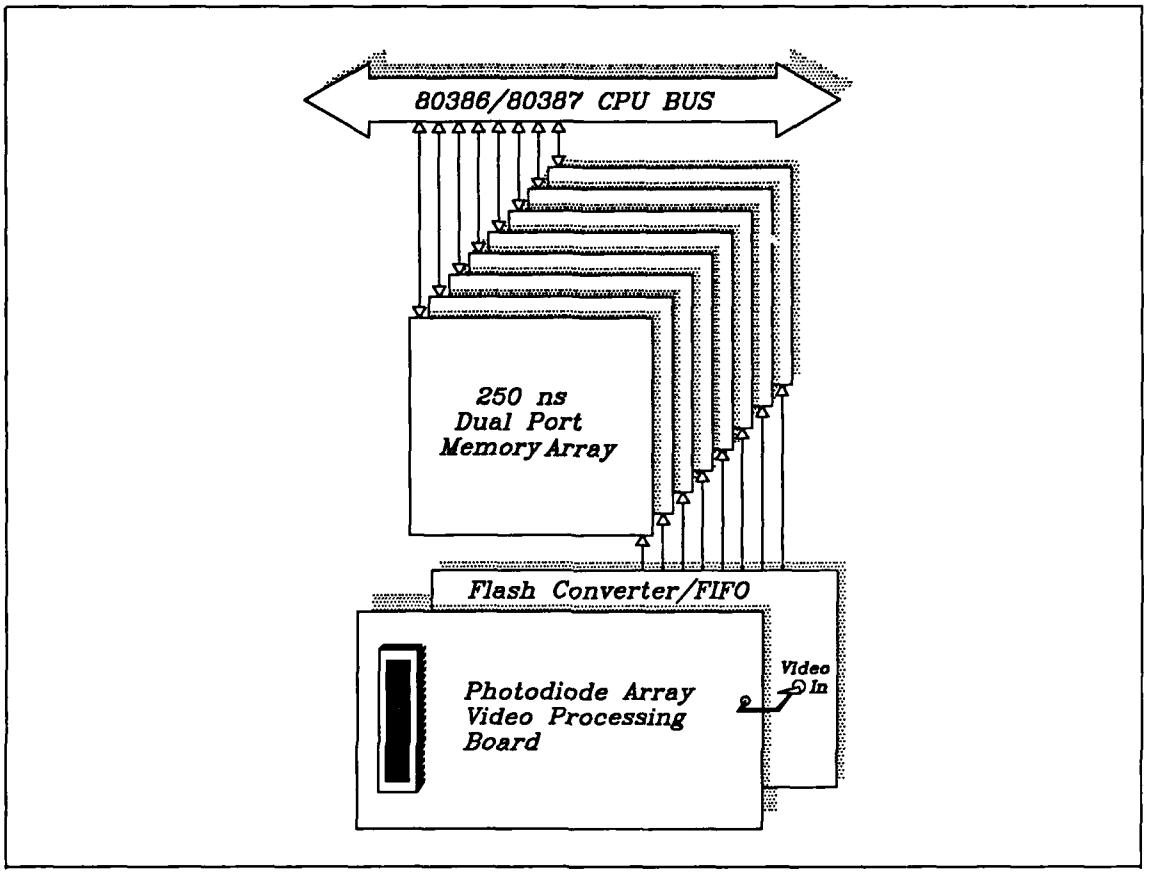

Figure 5

data to the remote memory array. Control signals from the memory array board can reset the PDA subsystem and initiate a video frame conversion. Once initiated, a video frame is digitized and loaded into the on-board FIFO without intervention of the host computer. The FIFO can hold an entire video frame in the event that transfer to the computer memory is delayed. As long as any data is present in the FIFO, the PDA subsystem will post a service request to the dual port memory array. Once transfer is complete, the system stands by for another conversion command, although a second conversion can begin before the FIFO is emptied, as long as data transfer is proceeding faster than frame conversion.

The memory array is a dual port configuration capable of accepting data in excess of the 5 million bytes/sec data rate possible with the photodiode array. Fully populated, this board has an 8 megabyte capacity. It is presently loaded with 2 megabytes. The board logic includes several I/O commands for control of the PDA subsystem. During idle time (that is, no video frame conversion/transfers are in progress) the board appears as a standard 375 ns system memory resource. The

location of this memory in the computers address space is freely selectable. Whenever the PDA subsystem posts a service request, 8 bit transfers will be made from the remote board as fast as possible. Logic on the memory board actively arbitrates between processor memory requests and the transfer of data from the PDA subsystem. Priority is given to processor memory requests, although at least one remote data transfer will occur between each processor memory cycle. Whenever the procesor is not accessing memory on the dual port board, the PDA subsystem will have unlimited access to the memory array, with a transfer occuring every 125 ns, for a net 16-bit transfer every 250 ns. Several companies generously contributed to this project. The memory was a gift from Micron Technologies, FIFO's were contributed by Integrated Device Technology, and National Semiconductor donated several of the logic components in the system.

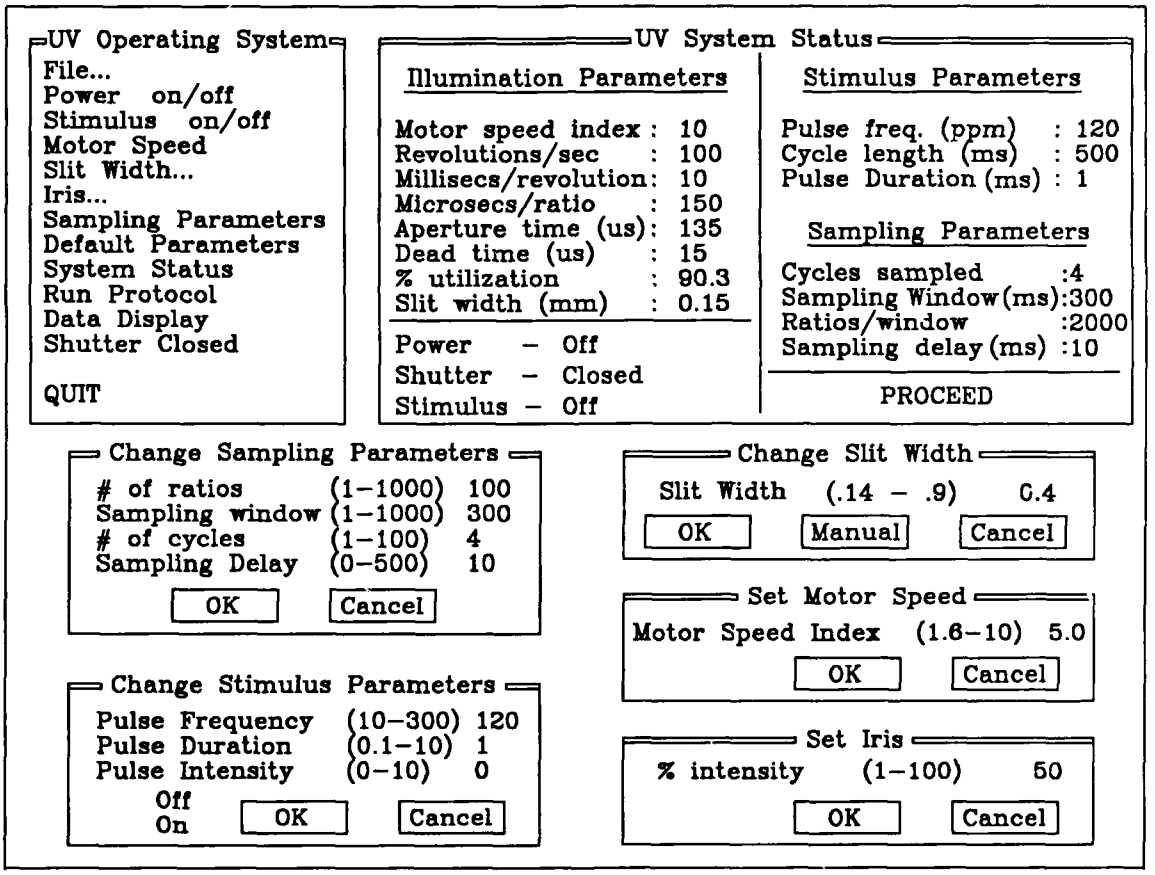

Figure 6

Computer software operating system: We have developed a software operating system (UVSYS) that features a graphical user interface for operator interaction. UVSYS provides comprehensive control over the microfluorimeter system, the electronic stimulator and all aspects of data acquisition. At the onset of each

experiment the operator composes an appropriate protocol from a system of interactive menus that set system parameters, exercise internal calibration procedures, select stimulus characteristics, and set data input parameters. Once a protocol has been defined it may be launched by aim-and-shoot mouse selection. Execution of the protocol is completely automatic. Error detection and handling is provided. Protocols may be changed at any time as dictated by the progress of the experiment. The general characteristics of UVSYS may be gleaned from the collection of menus shown in figure 6. The program was written in Quick Basic with the addition of a few assembly code fragments to expedite the highly time critical data acquisition routines.

DISTRIBUTION LIST

Program on Macromolecular and Cellular Effects of Pressure

INVESTIGATORS

Dr. Rodney L. Biltonen
Dept. of Pharmacology
University of Virginia
Charlottesville, VA 22908

Dr. P. A. George Fortes
Department of Biology, C-016
University of California
La Jolla, CA 92093

Dr. Hans Frauenfelder
Loomis Laboratory
University of Illinois
1110 West Green Street
Urbana, IL 61801

Dr. Perry M. Hogan
Department of Physiology
State University of New York
Buffalo, NY 14214

Dr. Thomas F. Murray
College of Pharmacy
Oregon State University
Corvallis, OR 97331-3507

Dr. Richard B. Philp
Dept. of Pharmacology & Toxicology
The Univ. of Western Ontario
Medical Sciences Building
London, Ontario, Canada N6A 5C1

Dr. Shalom R. Rackovsky
Department of Biophysics
University of Rochester
School of Medicine and Dentistry
602 Elmwood Avenue
Rochester, NY 14642

Dr. Lou Reinisch
Laser Biophysics Center
F. Edward Hebert School
of Medicine
4301 Jones Bridge Road
Bethesda, MD 20814-4799

Dr. Joseph F. Siebenaller
Dept. of Zoology and
Physiology
Louisiana State University
Baton Rouge, LA 70803-1725

Dr. Anne Walter
Dept. of Physiology and
Biophysics
Wright State University
Dayton, OH 45435

Dr. William B. Weglicki
Division of Experimental
Medicine
Ross Hall, Room 409
2300 Eye Street, N.W.
Washington, DC 20037