[19] Video-Rate Confocal Microscopy

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Introduction

The ability to observe cell function through the light microscope is a simple and direct method for obtaining a wealth of experimental information. The versatility of this approach has been greatly extended by the development of a diversity of fluorescent reporter dyes and molecular probes. Cell activities involving changes in ion concentrations (e.g., Ca^{2+} or pH) or the expression of proteins can be readily observed. However, a constant challenge is the requirement for high resolution both in the spatial and temporal domains. Typically, fluorescent molecules that are outside the plane of focus reduce image contrast and resolution. Thus, specimens with substantial tissue thickness or depth (i.e., almost everything except cultured or thin layers of cells) are difficult study with wide-field microscopy. Fortunately, confocal laser scanning microscopy (CLSM) provides one solution to this problem. With CLSM, a small aperture rejects the out-of-focus light and the final image represents a thin slice through the specimen with greatly improved axial resolution. However, a disadvantage of CLSM is that it requires that the specimen is, in general, illuminated with a scanning point of light. This has the consequence that the time needed to create an image is governed by the time to scan the specimen and the time or sensitivity of the instrumentation to detect fluorescent light from a small point source. Although most modern confocal microscopes employ a laser to produce a high-intensity point source for the excitation illumination (and thereby increase the amount of fluorescence emitted), the time taken to acquire an image can be relatively long. A slow acquisition time is unimportant when studying fixed or slowly changing specimens but precludes the study of many rapid biological processes.

Fast [30 frames per second (fps) or video-rate] confocal microscopes have been previously manufactured, but unfortunately, the cost of these microscopes was usually beyond the budget of individual investigators. These microscopes included the Nikon (Melville, NY) RCM 8000, a system that reflects a design described in detail by Tsien and Bacski[1]; the Noran (Middleton, WI) Odyssey; and the Bio-Rad (Hercules, CA) RTS2000. A limited number of sales of these high-speed confocal microscopes may explain the withdrawal from the market of one of the leading manufacturers (Noran). A consequence of this withdrawal will be the lack of support for some users—a situation to be avoided. One way to

[1] R. Tsien and B. J. Bacski, *in* "Handbook of Biological Confocal Microscopy" (J. B. Pawley, ed.), p. 459. Plenum Press, New York, 1995.

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circumvent all these problems and to acquire a high-speed confocal microscope, at an affordable price and with the assurance of continued maintenance, is to build it yourself.

Contrary to what might be expected, this requires relatively little experience in optics, electronics, or programming. The CLSM featured here is based on the specifications described by Callamaras and Parker[2] and represents the first microscope built (independently, but with considerable help) to that design by another investigator. The details of the original design are reiterated here, together with detailed practical instructions, diagrams for construction, and extensive appendices listing parts and supplies. In addition, several improvements are described, including the ability to correct, in real time, the image distortion arising from the variation in scanner speed, and to record (and play back) large numbers of images directly to (and from) a computer hard drive at both 30 (400 lines ×420 pixels) and 60 (200 lines ×420 pixels) frames per second.

Design Considerations and Methods

Although the history and details of confocal microscopy design cannot be addressed in this article, these are extensively reviewed in the text *Handbook of Biological Confocal Microscopy*.[3] This book is recommended for studying the practical issues of confocal microscopy.

Microscope Selection

Basic Microscope. An inverted microscope is the preferred choice for the construction of the CLSM because this type of microscope usually has an optical or camera port near bench level, which facilitates the integration of optical components with the microscope (Figs. 1 and 2). The choice between a 160-mm tube-length microscope and an infinity-corrected optical microscope is less critical. Both the Nikon Diaphot 200 (160-mm tube length) and the Olympus (Melville, NY) IX70 (infinity-corrected optics) have been successfully used as the base for a CLSM.

Image Redirection. The projection of an image to the optical side port is usually achieved by redirecting the light from the objective with an internal prism. The options available include redirecting 100% of the light so that the specimen can no longer be seen via the eyepieces or redirecting 80% of the light so that the specimen can be observed simultaneously via the eyepieces. Because the excitation light follows the same pathway as the emitted light in the CLSM, the properties of the redirecting prism will also influence the excitation illumination. As a result,

[2] N. Callamaras and I. Parker, *Cell Calcium* **26,** 271 (1999).
[3] J. B. Pawley, ed., "Handbook of Biological Confocal Microscopy." Plenum Press, New York, 1995.

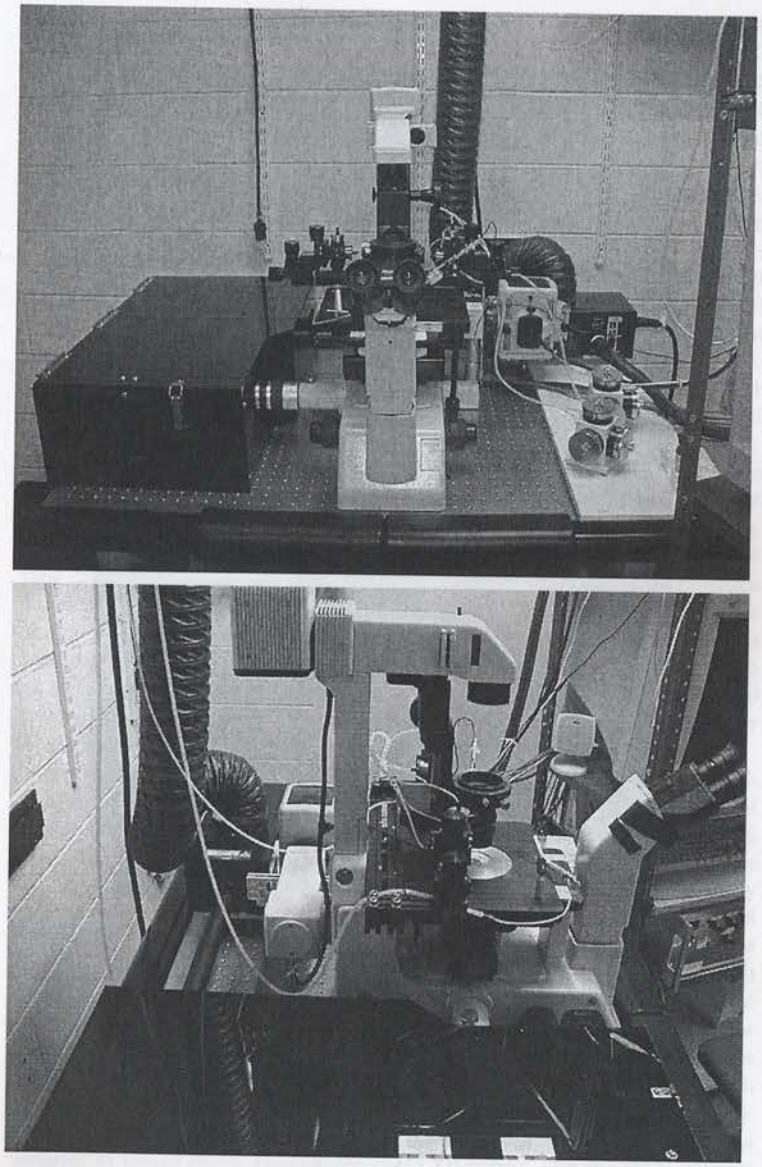


FIG. 1. A front view (*top*) and side view (*bottom*) of the confocal scanning laser microscope, illustrating the basic layout of the instrument on an air table. The black box contains the optical components. The flexible hose serves to exhaust cooling air to a ceiling air-conditioning vent.

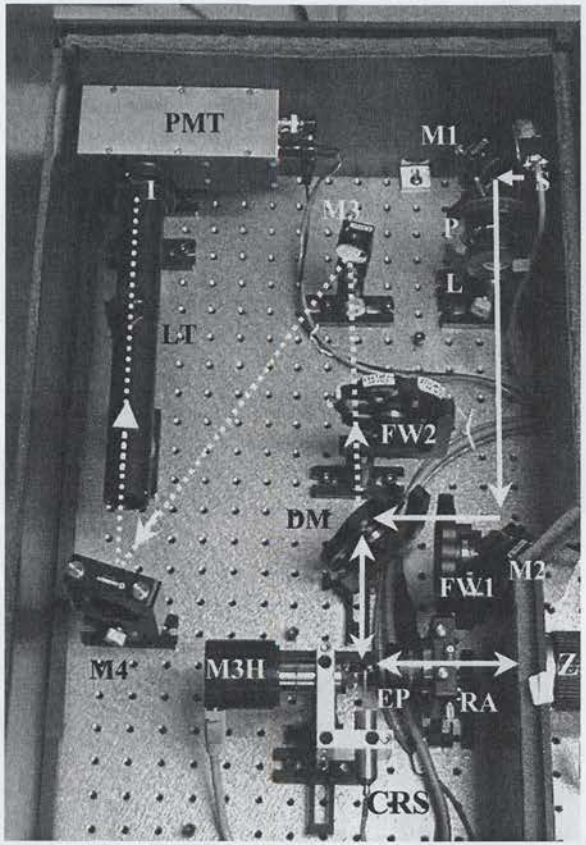


FIG. 2. A top view of the layout of the optical components and pathway for the excitation and emitted light in the CLSM. The solid white line indicates the path of the excitation argon laser light (488 nm). The laser enters the system through the shutter (S) and is reflected by mirror 1 (M1) to pass through the polarizer (P) and concave lens (L). The light is reflected by mirror 2 (M2), through filter wheel 1 (FW1) to the dichroic mirror (DM). The mirrors of the vertical scanner (M3H) and horizontal scanner (CRS) reflect the light through the eyepiece (EP) and rectangular aperture (RA) into the zoom adapter (Z) of the microscope. The emitted light (dotted line, >510 nm) initially follows the path of the excitation light until it reaches the DM. The emitted light passes through the DM and filter wheel 2 (FW2) to mirror 3 (M3) for reflection to the photomultiplier (PMT) via mirror 4 (M4), the light tube (LT), and confocal aperture (I). The optics are surrounded by a black Plexiglas box.

the choice of prism must be considered in view of a number of advantages and disadvantages. By redirecting all the light, the instrument will be most sensitive because all the excitation light reaches the specimen and all the emitted light can be monitored. In addition, stray light from the eyepieces is never a problem. However, with this approach, the alignment and location of the area or line being scanned by the system are more difficult to determine. By contrast, the simultaneous viewing of the specimen and scan area greatly facilitates alignment and specimen selection. A simple solution would be to have both options of 80 or 100% redirection available. Although we have not implemented this solution, the Nikon Diaphot 300 is equipped with this option. It is likely that some fine adjustments to the alignment would be required when switching between prisms.

SAFETY NOTE. If any laser light can reach the eyepieces, a safety barrier filter must be installed in the binocular microscope head. A long-pass 520-nm filter will block the 488-nm laser line but will transmit the fluorescent light.

Conventional Fluorescence Microscopy. The ability to quickly find the region of interest in the specimen is critical for successful observations and an initial search is facilitated by a wide field of view. Unfortunately, a wide field of view is not compatible with the small scanning area of the CLSM, but a wide field of view can be obtained by viewing the specimen by conventional epifluorescence illumination, using a mercury (Hg) or xenon (Xn) bulb. The excitation wavelength of light is directed to the specimen by inserting a standard filter cube carrying a band-pass excitation filter (485 nm) and dichroic mirror (505 nm) together with an appropriate emission filter (520 nm). Once the area of interest is found and centered in the field, the optics are easily changed for CLSM by sliding the filter block out of the way.

Phase-Contrast Microscopy. When examining fluorescent specimens with CLSM, it is often essential to obtain a nonfluorescent or transmitted light image of the specimen. This allows the spatial orientation and organization of the specimen to be interpreted. From our experience, the study of different tissues emphasizes the variability in this requirement. The study of calcium signaling in relatively large oocytes, which have a homogeneous cytosol, has little need for structural images. By contrast, the study of intercellular calcium signaling in multicellular systems cannot be achieved without structural information. Although the current CLSM design does not incorporate the acquisition of transmitted confocal images, this would be possible by adding a transmitted light detector after the condenser lens. An alternative approach is to make use of the conventional optics of the microscope. The Diaphot 300, like many inverted microscopes, has an additional camera port that can be selected. With a long working distance phase-contrast condenser and phase-contrast objective, phase-contrast images can be obtained with a separate 35-mm or charge-coupled device (CCD) camera. Use of a phase-contrast objective does not appear to cause any obvious degradation of the confocal image.

Microscope and Optical Mountings. It is advisable to establish the system on an air-suspended optical table, not only to isolate the system from external

TABLE I
MAJOR WAVELENGTHS OF EXCITATION LIGHT PRODUCED BY DIFFERENT LASERS AND CORRESPONDING FLUORESCENT DYES

Excitation wavelength (nm)	Dye	Laser
364	Indo-1	UV argon
488	Fluo-3, Fluo-4	Argon, air cooled (argon–krypton)
	FITC	
	Oregon Green	
	Fura Red	
	GFP	
514	YFP	
543	Cy-3	Green He–Ne
	TRITC	
	DiI	
568	TRITC	Krypton (argon–krypton)
	DsRed	
594	Texas Red	Yellow He–Ne
647	Cy5	Krypton (argon–krypton)

vibration, but for ease of aligning and integrating optical components (Figs. 1 and 2). A common selection is a table with a stainless steel top with threaded holes (0.25 inch, 20 thread) centered on a 1-inch grid. The alignment of optical components is achieved with a range of adjustable mounting posts that are available from all the major optical companies. Because the exact height requirements for each component vary, it is advisable to have a selection of posts and post holders ranging from 1 to 4 inches available. The posts screw directly into the air table or can be mounted on an adjustable base when their alignment does not coincide with the tabletop hole pattern.

Laser Selection

A major component of the CLSM is the excitation laser. Consequently, the choice of laser or lasers should be considered carefully in view of future experimental goals. The development of a wide range of fluorescent dyes, which can be used to detect a variety of biological molecules, provides the means for a versatile instrument.[4] Although it is not feasible to design an instrument for all possibilities, a wide range of options can be accessible if the major laser line excitation wavelengths are available. These major wavelengths include 364, 488, 514, 543, 568, 594, and 647 nm. Some of the lasers that produce the appropriate excitation lines for various dyes are summarized in Table I.

[4] R. Y. Tsien and A. Waggoner, *in* "Handbook of Biological Confocal Microscopy" (J. B. Pawley, ed.), p. 267. Plenum Press, New York, 1995.

For the basic system, a single air-cooled argon laser provides laser lines of 488 and 514 nm, which can be used to excite a variety of dyes. However, if multiple dye experiments are required, the choice of an argon–krypton laser would add lines at 568 and 647 nm. Alternatively, a second green helium–neon laser can be incorporated to provide a line at 543 nm. The choice of lasers depends mainly on the application and resources available. It should also be noted that certain laser lines fade faster than others in mixed gas lasers. As a result, separate lasers for each line may be more cost effective.

The argon laser (maximum output, 100 mW) is frequently operated at about 25 mW (in stand-by mode) to conserve tube life. Therefore, a less powerful laser can be purchased to save costs. However, the argon laser model used is compact and has the advantage of having some reserve capacity for weak signals. The laser is purchased with a cooling fan unit. Because the laser generates a considerable amount of heat, it is advantageous to plumb the exhaust vent into the air conditioning system. To lessen noise, a mounting location above the ceiling tiling is also advantageous. A switched 20-A, 120-V power supply is required, and is preferably dedicated to the laser alone.

Laser Scanning Mechanism

The scanning of the laser beam across the specimen is achieved with two scanning mirrors mounted at right angles. For alignment, each scanner is mounted in a simple bracket so that the mirror can be moved along its axis as well as rotated before being clamped in place (Figs. 3 and 4). The complete assembly is mounted on a rail for alignment. The horizontal scan is generated by a mirror mounted on a resonant scanner that oscillates at a fixed frequency of about 8 kHz (Counter Rotating scanner, CRS; GSI Lumonics, Bedford, MA). This mirror rotates through an angle (up to 26°) with an angular velocity that reflects a cosine function. The fact that the mirror moves in a predictable manner means that a software algorithm can be performed to correct the image distortion that results from the change in mirror velocity (see Software for Correction of Image Distortion, below). The pair of controller boards supplied with the scanner provide all the necessary electronics for the operation of the scanning mirror. The user needs only to supply power and a control voltage to regulate the magnitude of the scan angle. The magnitude of the scan angle determines the horizontal magnification. These boards also provide the horizontal synchronization signal.

The vertical scan is generated by a second mirror mounted on an M3H scanner (GSI Lumonics) that rotates in response to a sawtooth waveform at 30 Hz. The mirror attached to the M3H is mounted "off axis" at the end of a short extension arm or paddle. This arrangement minimizes the amount of beam rotation induced by scanning mirrors.[2] The M3H scanner is also controlled by an independent driver board (OATS driver) and requires only a power supply and two input voltages (a sawtooth waveform and an offset) for position control. GSI Lumonics

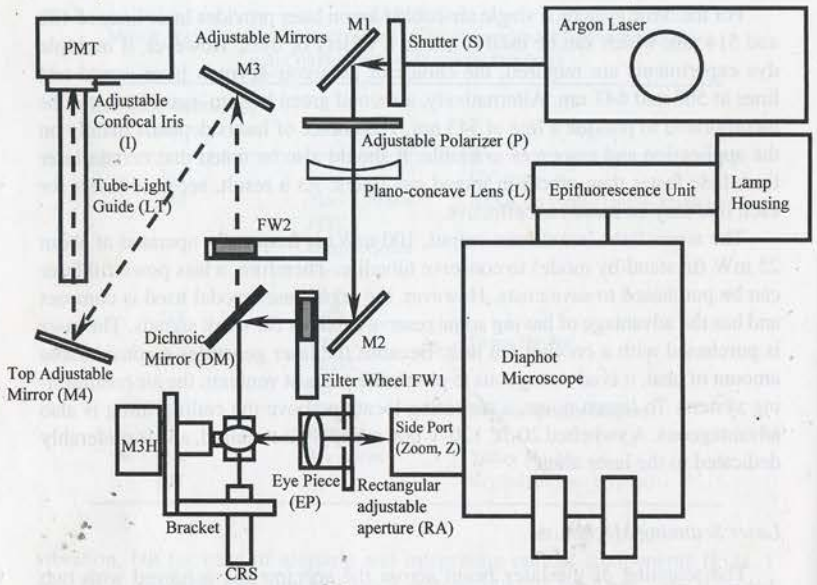


FIG. 3. A schematic layout of the CLSM, showing the positioning of the microscope, laser, and components shown in Fig. 2. Abbreviations are the same as for Fig. 2.

recommends the replacement of the M3H scanner with an improved M3S scanner and "MiniSax" driver. These components have similar characteristics and should be easily substituted for the M3H scanner in the design. The major difference between the M3H and M3S scanner is that the scan angle amplitude control is reduced from ±5 to ±3 V.

When purchasing the M3H scanner (or M3S), it is important to request that the scanner be set up or "tuned" with its driver board and with the paddle mirror attached for a 1- to 3-V, 30- to 60-Hz sawtooth waveform. This minimizes resonance frequencies from distorting the scan. It is also important to note that the control cable from the OATS board to the M3H scanner is not a 15-pin straight-through cable (wiring is not pin to pin). However, a 15-pin cable can be used to extend this cable. Because the documentation provided with these boards is sparse, we indicate pin locations and connections in Fig. 8.

Photomultiplier Tube

The detector used for the CLSM is a photomultiplier tube (PMT), which produces an analog voltage that is proportional to the light intensity. Although the

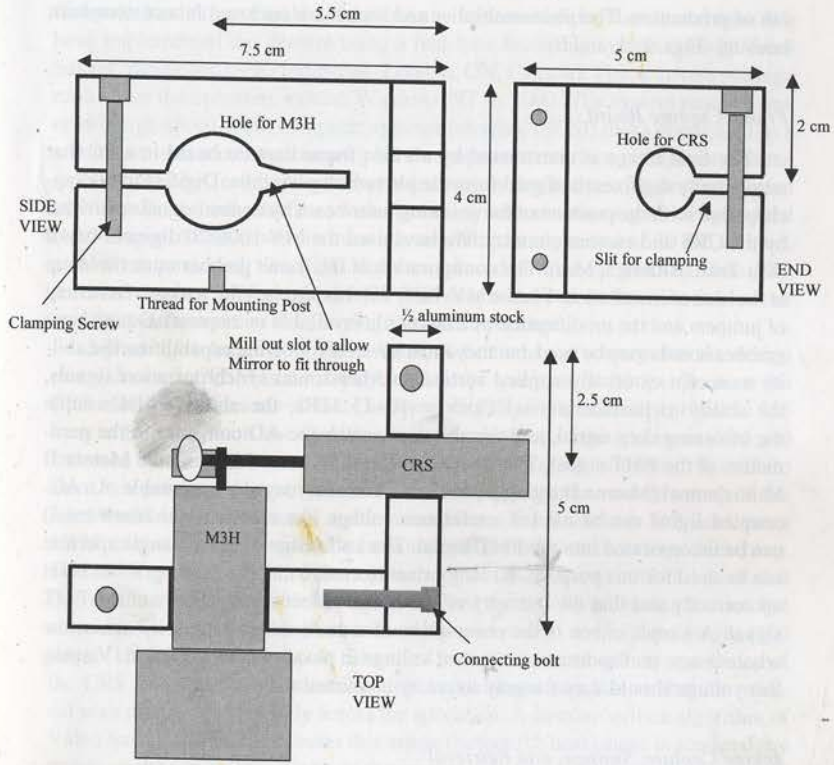


FIG. 4. A detailed construction plan of the mounting bracket that carries the CRS and M3H scanners.

quantum efficiency of photomultipliers is lower than that of solid-state detectors (e.g., avalanche photodiodes), they are still the best detector for use at the high photon count rates ($>10^8$ photons s^{-1}) required for video-rate imaging. Because the wavelength of the light emitted from the specimen is between 500 and 650 nm, the photomultiplier tube was selected to have maximal sensitivity at these wavelengths (R3896; Hamamatsu, Bridgewater, NJ). This photomultiplier replaces the tube used in earlier designs.[2] The photomultiplier is mounted in a specialized socket (C7247-01) that also contains the amplification circuit to provide a positive DC voltage. The amplifier has a bandwidth of DC-5 MHz and matches the required filtration. The high-voltage power supply (C4900-00) is a circuit board component that is incorporated into the system control box. The socket and power supply replace components previously described[2] because the old components are now

out of production. The photomultiplier and socket are enclosed in a custom-built housing (Figs. 2, 3, and 6).

Frame Capture Board

The final image is constructed by a video frame capture board in a PC that sequentially digitizes the signal from the photomultiplier tube. Digitization is synchronized with the position of the scanning laser beam by control signals provided by the CRS and custom circuitry. We have used the MV-1000-20 digitizer board (Mu-Tech, Billerica, MA). The configuration of the frame grabber must be set up at the time of installation. For the MV-1000-20, this requires the setting of a variety of jumpers and the modification of a driver file (available on request). Other frame grabber boards may be used, but they must have the following capabilities: the ability to accept externally applied vertical and horizontal synchronization signals, the ability to provide a pixel clock at 10–15 MHz, the ability to DC-couple the incoming data signal, and the ability to match the AD converter to the parameters of the PMT signal. The Raven (BitFlow, Woburn, MA) and the Meteor II Multichannel (Matrox Imaging, Dorval, PC, Canada) may also be suitable. An AC-coupled signal can be used if a reference voltage (for clamping the black level) can be incorporatèd into the PMT signal. The knife edge of the rectangle aperture can be used for this purpose. It is important to ensure that the frame grabber is set up correctly and that the intensity of the image reflects the intensity of the PMT signal. A simple check is the observation of a uniform gray intensity across the whole image in response to a constant voltage in place of the PMT input. Varying this voltage should vary the gray intensity in a linear fashion.

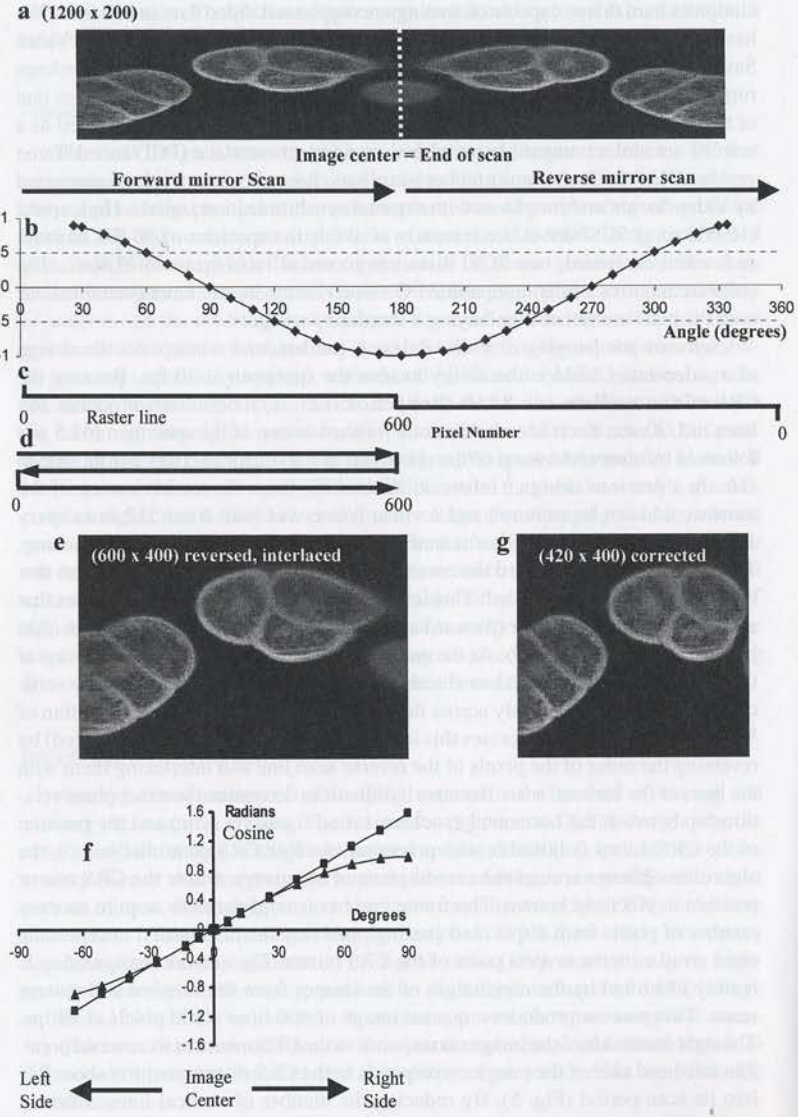
Image Capture, Storage, and Retrieval

A major consideration of all imaging systems is the method of storing and viewing the acquired images. The reason for the current CLSM design was the need to collect images with good temporal resolution (30–60 fps). However, the consequence of this design is that large numbers of images are generated in short periods of time. Recording images to a video cassette recorder (VCR) is the simplest option. Although analog [video home system (VHS)] recorders have many disadvantages, the new generation of digital video (DV) recorders allows high-quality frame-by-frame replay, and can be digitally interfaced to a PC through an IEEE 1394 (FireWire) adapter. A similar approach is to use an optical memory disk recorder to record images. This has the advantage of random access to individual frames, but the costs and availability of recording disks are becoming prohibitive.

Software for Real-Time Recording to Hard Drive. A better solution to the problem of image storage and management has become possible with the availability of

computer hard drives capable of writing uncompressed video data in real time. We have implemented this feature using a real-time recording system called "Video Savant" developed by IO Industries (London, ON, Canada). This software package runs under the operating system Windows NT or 2000. The system requires one or two high-speed small computer systems interface (SCSI) disks configured as a volume set and a compatible peripheral component interface (PCI)-based frame grabber. The Mu-Tech frame grabber board was chosen because it is both supported by Video Savant and interfaces with external synchronization signals. High-speed (10,000 rpm) SCSI drives are currently available in capacities of 30 GB or more and, when combined, two SCSI disks can record at rates up to 45 MB/sec. The software requires a fully compatible PC mother board and we have assembled our own PC platform rather than buying a standard package.

Software for Imaging at Video Rates. A fundamental concept for the design of a video-rate CLSM is the ability to scan the specimen at 30 fps. Because the CRS mirror oscillates at ~8 kHz (line period, 125 μs) it nominally produces 265 lines in 1/30 sec. Each line consists of a forward sweep of the specimen (62.5 μs) followed by a reverse sweep of the specimen as the mirror oscillates from side to side. In a previous design,[2] information resulting from the reverse sweep of the mirror could not be captured and a video frame was built from 512 lines every 1/15th of a second. The reverse scan would still contribute to specimen bleaching. Therefore we have exploited the reverse scan of the mirror to collect data so that the frame rate can be doubled. This is achieved by initially collecting frames that are twice the normal width (forward and reverse sweep) and half the height (200 lines $\times$1200 pixels) (Fig. 5). At the end of both the forward and reverse sweep of the CRS mirror, the vertical sawtooth is incremented one line so that the vertical scan progresses smoothly across the specimen. A custom-written algorithm of Video Savant software processes this image (before the next image is acquired) by reversing the order of the pixels of the reverse scan line and interlacing them with the lines of the forward scan. Because it difficult to determine the exact phase relationship between the horizontal synchronization signal (H sync) and the position of the CRS mirror (adjustable with potentiometer R48 CRS controller board), the algorithm operates around the central plane of symmetry, where the CRS mirror position is precisely known. The frame grabber is configured to acquire an even number of pixels from a specified starting pixel that can be adjusted to center the pixel array with the reverse point of the CRS mirror. The correct configuration is readily identified by the registration of the images from the forward and reverse scans. This process produces a normal image of 400 lines $\times$600 pixels at 30 fps. The right-hand side of the image corresponds to the CRS mirror at its reversal point. The left-hand side of the image corresponds to the CRS mirror position about 24° into its scan period (Fig. 5). By reducing the number of vertical lines collected (determined by the output selected from the binary counter; Fig. 8), image rates of 60 fps (200 lines) or 120 fps (100 lines) can be achieved. In the extreme case,



only a single horizontal line is collected (line-scanning mode); the vertical scan mirror is stationary and used only to select the position of horizontal line.[5]

Software for Correction of Image Distortion. Because rapid scanning is achieved with a resonant scanner, the angular velocity of the scan rate is not constant and varies as a cosine function. At the extreme ends of the scan, the mirror is instantaneously stationary. As the mirror moves through the scan it reaches a maximum velocity at the central position before slowing to zero at the opposite end of the scan. To digitize the image, frame grabbers commonly employ a pixel or sampling clock that has a constant interval and this assumes a linear translocation of the beam. As a result, the image appears stretched at the edges because the actual sampling occurs at almost the same position while the pixel position is assumed to be progressing linearly. A simple solution to this problem is to record or utilize data from the central 66% of the mirror scan, where the velocity of the mirror is almost linear.[2] In this case, the blades of the rectangular aperture are used to mask the area of interest in order to protect the specimen exposed to the extremes of the scan line from bleaching. Toward each end of the scan, the dwell time of the laser for each pixel is rapidly increasing. This translates into a longer illumination time and thereby increases the risk of photobleaching.

An alternative way to use a greater extent of the scan is to perform a spatial correction for each pixel. We have developed an algorithm (Fig. 5), which is now included in Video Savant, and works in real time for distortion-free viewing and image recording. This algorithm is not hardware dependent and will work for

[5] I. Parker, N. Callamaras, and W. G. Weir, *Cell Calcium* **21,** 441 (1997).

FIG. 5. Procedures for obtaining a corrected final image, using a nonlinear sinusoidal resonant scan mirror. (a) The initial unprocessed image acquired by the MV-1000-20 frame grabber consists of 1200 pixels ×200 lines. Each line is constructed from the forward and reverse scan of the CRS mirror to generate a mirror-image pair of the specimen. (b) The velocity profile of the CRS mirror as it moves forward and in reverse across the specimen to form the image seen in (a). The collection of image pixels is initiated and terminated when the mirror is 24 or 336° into its oscillatory period. As a result, the distortion of the image is most noticeable toward the center of the image shown in (a). (c) To avoid scanning the same section of the specimen, the vertical positions of the forward scans and the reverse scans are incremented by a single linewidth each time the mirror changes direction. (d) The effective raster pattern used to form the real image is obtained by reversing the order of the pixels in the reverse scan and interlacing them beneath the pixels in the forward scan. Numbers represent the real pixel location. (e) The resulting image after pixel reversal and line interlacing. The image is 600 pixels wide and 400 pixels high. (f) The relationship of the actual velocity profile (triangles) and the assumed velocity profile (resulting from a constant pixel clock frequency, squares) of the CRS mirror as it scans the specimen to form the image shown in (e). Image distortion is corrected by transforming the assumed pixel location to the real pixel location. (g) The resulting distortion-free image of that shown in (e). The corrected image has fewer pixels per line because most of the pixels toward the ends of the scan of the distorted image are duplicates.

images of any pixel width. The algorithm is applied after image reversal and uses the right-hand side of the image (reversal point of the CRS mirror) as a reference point (Fig. 5f) in order to determine the center pixel of the image. From the center of the image (in both directions),

$$P_{\text{new}} = P_{\text{old}}/\text{correction factor}$$

where the correction factor is the velocity factor of the pixel position or the angular position (from the center, in radians) [ranges from 1 at the center of the image to 1.57 (90°) at the end of the sweep].

Software for Image Acquisition, Analysis, and Presentation. The major impetus for incorporating commercial software for image recording was to avoid software development in the laboratory. While this can have many advantages for customization, software development is difficult and time consuming. Video Savant has solved most of the problems associated with rapid image acquisition and has provided a simple user interface. In addition, this company has been willing to write custom software to perform image reversal and distortion correction. To record images, all that is necessary is to define a file of the appropriate size and highlight the number of frames to be recorded. Clicking a control panel that mimicks a VCR initiates image acquisition. Image acquisition can be either continuous at 30 fps or discontinuous with single frames (time-lapse), sequences of frames, or averaged frames being recorded in response to a synchronization signal that also controls the laser shutter. The synchronization pulses can be generated by Video Savant and the Mu-Tech board or by an external stimulator (Fig. 9). Playback follows the same procedure with the options of looped play back and different playback speeds. Playback can be performed at speeds faster than the acquisition speed, which greatly facilitates the recognition of changes in the image intensity. Video Savant has a number of image analysis tools including averaging and image division and subtraction.

Sequences of complete images or regions of interest can be exported in a variety of file formats. We have found that the most convenient file format is a tagged image file (TIF) stack. This format is fully compatible with NIH Image (Scion, Frederick, MD; free software for PC) and other analysis packages. Images can be pseudo-colored during acquisition or playback and can be saved as MPEG (moving picture experts group) movie files. Image sequences can be archived to CD-ROM (compact disk-read only memory).

Computer System

For Video Savant to work reliably, a compatible mother board is required. Use of the very latest chip sets may cause incompatibilities and it is advisable to install a tested, even if older, mother board. The major characteristics required are an Intel (Santa Clara, CA) BX support chip set for a Pentium III processor, and an advanced graphics port (AGP) slot. A mother board with the ability to hold up to

1 GB of memory is an advantage. We have chosen the ASUS mother board P3BF with an 800-MHz processor. This processor comes only in the FCPGA format and an adapter card is required to install it in the mother board (slot 1 type). The second major requirement is a high-speed SCSI controller and two high-capacity, high-speed SCSI drives. The fastest version for the SCSI is currently the Ultra 160. It is possible, with the increasing speed of SCSI drives, that only one drive will be required, but two will always give the advantage of increased capacity. A video graphics card by ATI is recommended because these cards have worked consistently. A 21-inch monitor is recommended because the microscope images will be viewed on this screen at a resolution of 1024 × 796. The operating system (OS) of choice is Windows 2000, to keep pace with software development and networking. This OS installs easily on the large-capacity drives. A CD-ROM or DVD-ROM (digital video disk-read only memory) or DVD-RAM (digital video disk-random access memory) drive is recommended to archive and back up images files. Most of the other computer components are not critical. In view of these special requirements, we recommend purchasing components separately and assembling the computer in the laboratory. The frame grabber board is installed in the computer in a PCI slot and its software drivers are installed according to the manufacturer. Alternatively, IO Industries will custom build, on request, a computer system with the frame grabber and Video Savant software installed.

Image Acquisition along Axial Plane

It is frequently required, especially in thick specimens, to examine the tissue at several levels. With thin optical sections produced at video rate by the CLSM, this simply requires a change in focus. However, the rapid reproducible excursions along the z axis, which are necessary to compare activity at different planes within a tissue, are unattainable by manual focus adjustment. It is therefore recommended that a piezoelectric focusing device (Polytec PI, Auburn, MA) be incorporated into the design. The sections at precise depths through the pollen grains shown in Fig. 11 were obtained with this equipment. The unit consists of a piezoelectric linear translator that attaches the objective to the microscope turret. The adapter raises the objective position by about 5/8 inch and this requires that the microscope stage be similarly raised. The incorporation of the adapter will slightly lengthen the optical path, and should be, if possible, completed before aligning other optics.

Microscope Construction

Optical Arrangement

The construction of the microscope is similar to that described by Callamaras and Parker.[2] Little fabrication is required because most components are mounted with optical supports or studs that screw into the air-table top. In this design, only a

mirror (M4). Repeat the process in order to align the beam on the photomultiplier aperture. Start with the aperture fully open and monitor the signal intensity with an oscilloscope. Once the beam is roughly aligned the signal can be maximized with the adjustment screws of the mirror (M4). Continue to maintain a maximal signal by moving the beam with the adjustment screws while the aperture is reduced in size, ensuring, throughout, that the reflective specimen remains in focus.

Step 6. Replace the foil slide with a pollen grain slide and focus on the grains. Connect the signal to the imaging board to see an image. Small adjustments to the illumination beam and emitted beam may be required to increase the brightness of the image and the sharpness of the image. A uniform layer of dye (e.g., 10 μM fluorescein, sandwiched between a slide and cover slip) should be examined to confirm even illumination.

Control Electronics

A single control box is recommended to house a variety of circuits and centralize the control functions. The circuits in the control box are as follows:

Photomultiplier power and amplification (Fig. 6)
Laser control and power regulation (Fig. 7)
Generation of timing signals for the M3H from the CRS (Fig. 8)
General power and shutter control (open, time lapse) (Fig. 9)

The construction of the electronics is relatively straightforward, requiring some machining of the box faceplates to accept switches, dials, and receptacles (Fig. 10, Appendices B and C) and the soldering of electronic components to PC boards. Several power supplies are required (± 5, ± 15, and ± 24 V) (Fig. 9). Independent power switches and LED (light-emitting diode) indicators are useful so that various parts of system can be operated in isolation.

Photomultiplier Circuit. The high voltage for the PMT is provided by module C4900-00 (Fig. 6). The module requires power and a 50-kΩ potentiometer to control the gain of the PMT by varying the applied high voltage. The PMT current signal is converted into a voltage by an amplification circuit built in the socket. A front panel switch controls the power to the PMT.

Laser Control. The argon laser is supplied with a 25-pin control card that plugs into the power supply (Fig. 7). For convenience of monitoring and adjusting the board, the laser control board is incorporated into the central control box. A 25-pin parallel cable connects the power supply of the laser to the control box. The controls on the laser board are simply extended to the front panel of the control box. A panel voltmeter is added to provide a digital readout of the operating power of the laser. The voltage applied to the meter is scaled with a resistor (voltage divider) so that 1 mV = 1 mW.

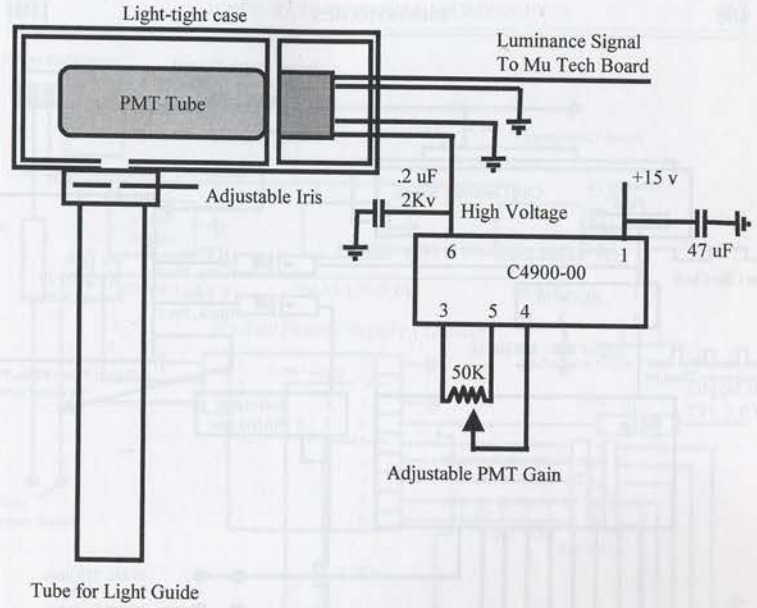


FIG. 6. Circuits associated with the photomultiplier, and layout of the photomultiplier/confocal aperture housing.

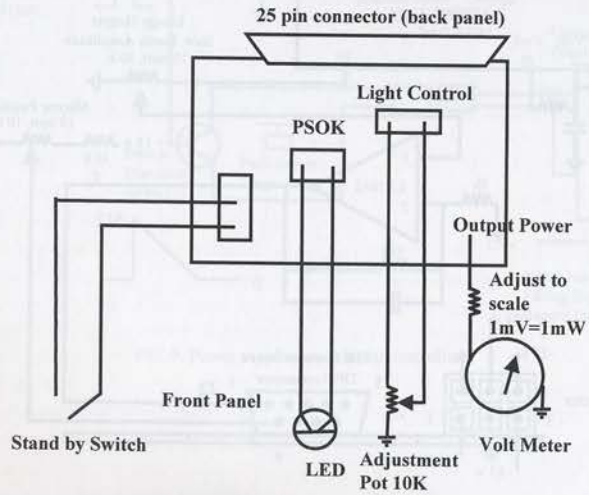


FIG. 7. The circuit controlling laser function from the front panel of the control box.

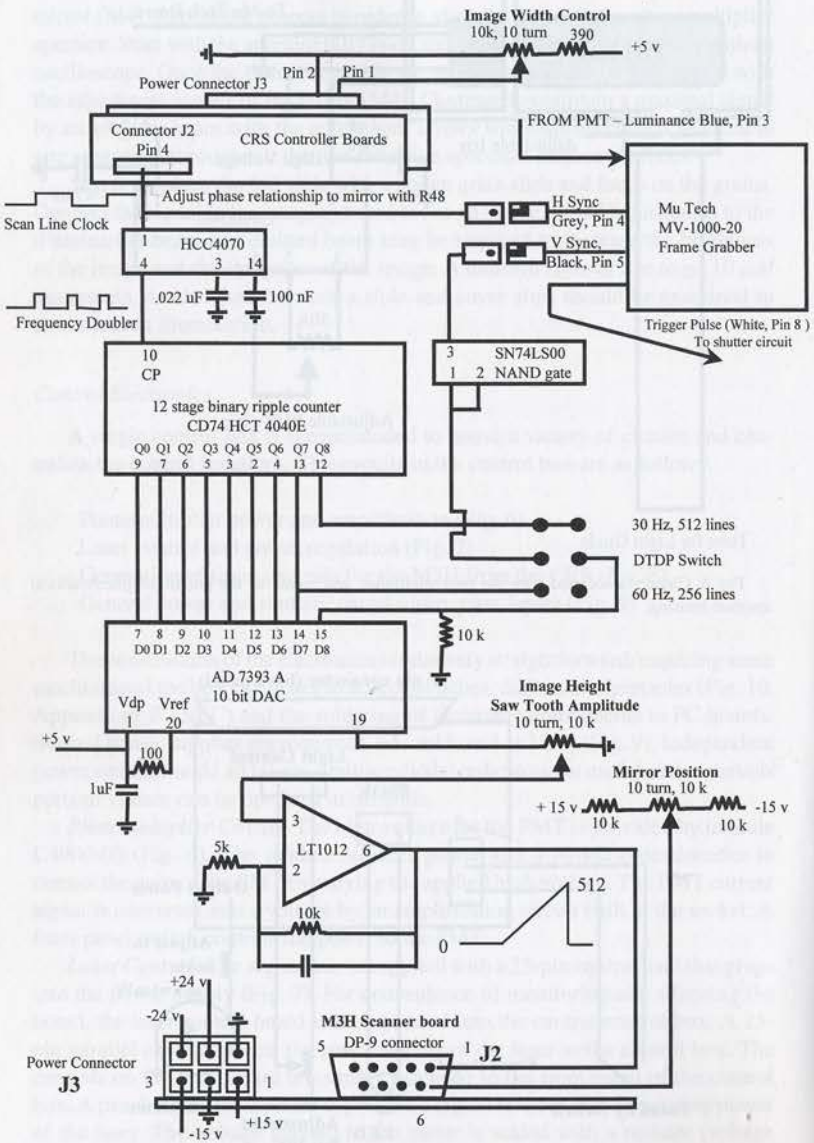


FIG. 8. Circuits generating the sawtooth ramp drive signal to the M3H scanner and horizontal (line) and vertical (frame) sync pulses to the frame grabber board.

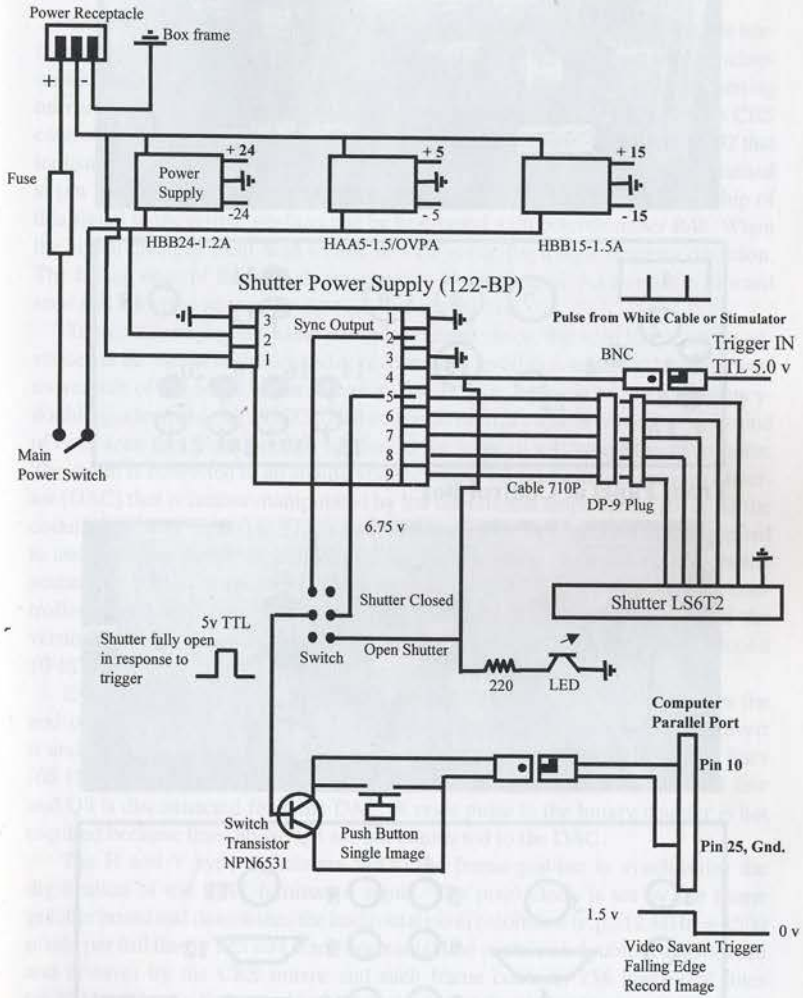
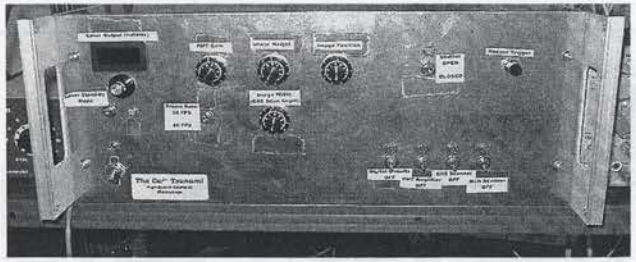
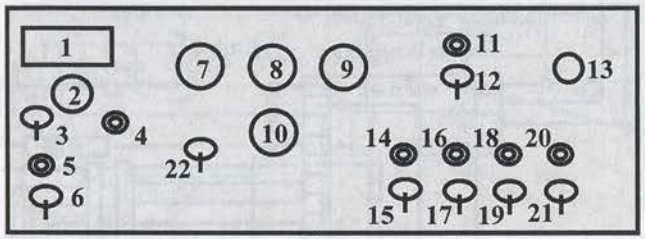
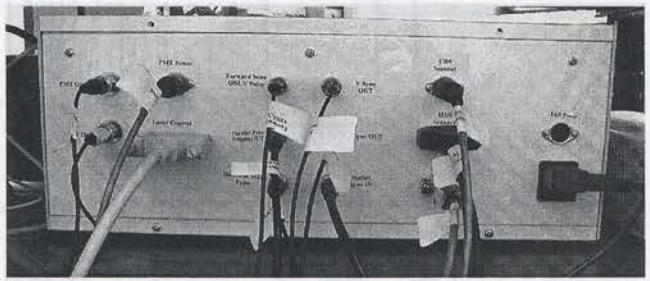


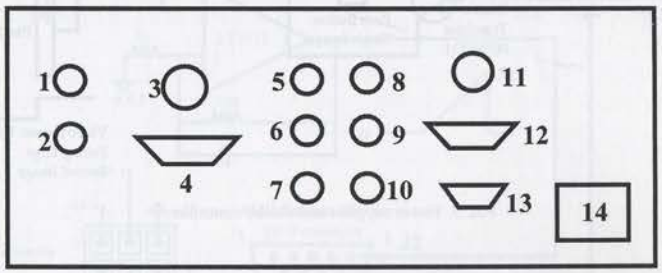
FIG. 9. Power supplies and shutter controller.





Front Panel of Control Box





Back panel of control box

FIG. 10. The layout of the front and back panels of the control box. Refer to Appendices B and C for identification of each component.

Generation of Timing Signals. The key circuit of the CLSM generates, the horizontal synchronization signal (H sync) of the CRS (Fig. 8), the vertical synchronization signal (V sync), and sawtooth waveform that drives the vertical scanning mirror. A separate front panel switch controls power for these circuits. The CRS control boards generate a square-wave timing signal from pin 4 of connector J2 that indicates the direction of the CRS mirror rotation. This signal can also be obtained at pin 8, connector J3 if jumper W1 is installed. The exact phase relationship of this signal to the mirror position can be fine-tuned with potentiometer R48. When the signal changes from high to low or vice versa, the mirror reverses direction. The falling edge of this signal serves as the H sync signal. As a result a forward scan and a reverse scan constitute one horizontal line.

To avoid scanning the same part of the tissue twice, the scan line must be advanced at the end of each forward or reverse scan and this is achieved by the slower movement of the M3H scanner mirror. The H sync signal is fed to a frequency-doubling integrated chip (HCC4070) and, as a result, a pulse is generated at the end of each scan line. Each pulse is applied to the input of a 12-stage binary counter. The count is converted to an analog voltage by the 10-bit digital-to-analog convertor (DAC) that is further manipulated by the operational amplifier LT1012. As the count progresses from 0 to 512, a sawtooth waveform is generated that is applied to and displaces the M3H scanner (J2, pin 1). In essence, each pulse advances the scanning position of the laser. The amplitude of the M3H displacement is controlled by a 10-kΩ potentiometer (front panel). An offset voltage to control the vertical or central position of the M3H mirror is provided (J2, pin 6) by a second 10-kΩ potentiometer (front panel) (Fig. 8).

Every 512 counts, line Q8 of the binary counter goes high and indicates the end of the current image. The signal is feed through an NAND gate to invert it and form the V sync signal. The selection of 512 lines (30 Hz) or 256 lines (60 Hz) per image is possible with a switch. Line Q7 serves as the control line and Q8 is disconnected from the DAC. A reset pulse to the binary counter is not required because lines above Q8 are not connected to the DAC.

The H and V sync signals are fed to the frame grabber to synchronize the digitization of the PMT luminance signal. The pixel clock is set by the frame grabber board and determines the horizontal pixel resolution (e.g., 12 MHz = 1500 pixels per full line in 125 μs). Each horizontal line contains a double scan (forward and reverse) by the CRS mirror and each frame contains 256 horizontal lines (at 30 Hz) (Fig. 8). Software is used to reconstruct the viewable image in real time (Fig. 5).

M3H Scanner Control Board. The M3H scanner control board requires a heat sink and should be bolted to the control case. A 15-pin flat ribbon cable is used to connect the M3H board (J1) to the back panel of the control box. The M3H board is connected to the M3H scanner with the custom cable (provided) and a 15-pin extension cable. Power to the M3H board is applied via the 6-pin plug J3. Control

signals are applied to J2 as described above. A front panel switch controls power to the M3H.

CRS Control Boards. CRS control boards [pixel clock board (PCB) and driver board (DB)] require heat sinks and are bolted to the control box. A flat ribbon cable (provided) connects the two boards together (PCB, J2–DB, J2). The board is connected to the scanner head via the back panel of the box and a 5-pin connector and cable attached to DB, J1. Additional flat ribbon cables are required for pins PCB, J1 (24 pin, data from this connector not used in the current configuration) and DB, J3 (16 pin). Power is provided through J3. The amplitude of the scan of the CRS is controlled by a variable voltage provided by a 10-kΩ potentiometer (front panel) applied to pin 2, J3 (Fig. 8). An additional resistor of 390 Ω is added, in series, for voltage protection (maximum, <5 V). A separate front panel switch controls power to the CRS.

Shutter Control. An independent, fully integrated power supply and controller (122-BP) is used for shutter (LS6T2) control (Fig. 9). For manual control of the shutter, a front panel switch is used. For automatic control, the shutter is placed in the closed position with the manual switch and a shutter trigger pulse (TTL, 5.0 V) to pin 4 of the controller results in the shutter opening. The generation of this trigger pulse is controlled from within Video Savant. When the shutter is 80% open, a synchronization pulse is generated at pin 5, and this is applied to a switch transistor. The activation of the transistor grounds the parallel port trigger of the Video Savant software. As a result, image acquisition is initiated when the shutter is fully open. However, to prevent the shutter closing prematurely, the duration of the trigger pulse must be slightly longer (one or two frame periods) than the time needed to record the required number of images. Closure of the shutter resets the system. Time-lapse recording is achieved by the trigger pulse frequency.

Results and Discussion

This version (MJS1.0) of the CLSM took approximately 1 year to assemble. However, much of that time was taken up in waiting for parts to be delivered, with the realization that, once they had arrived, other parts were still required. Following this experience, we have attempted to provide a full parts list that will allow investigators to order all the parts at once. Furthermore, additional time was required to implement a number of design improvements over the original model[2]; specifically the incorporation of software for forward and reverse scanning, the correction of image distortion, and the ability to save large numbers of images to hard disk. We consider the design to be now beyond the beta-testing stage and, if the detailed instructions provided here are followed, envisage that an investigator should be able to construct the CLSM within 4 months. Despite the modest cost of the components required for construction, the performance of the CLSM is comparable to that of commercial instruments costing hundreds of thousands of dollars.

Figure 11 demonstrates the ability of the CLSM to acquire a series of thin confocal sections at different planes. The specimens illustrated are "spiky" and lobed pollen grains that provide a convenient test for checking the operation of the CLSM and comparing its performance with other microscopes. The shape of each pollen grain is easily determined and the details of its inner structure are clear. The iris aperture easily regulates the extent of rejection of out-of-focus light so that image brightness and slice thickness can be optimized in the final image. A full comparison of images of pollen grains has been presented by Callamaras and Parker.[2]

The video-rate CLSM has been extensively used for imaging elementary intracellular calcium dynamics.[6,7] In conjuction with the photolytic release of inositol trisphosphate, the rapid localized release of calcium by clusters of IP_3 receptors or "Ca^{2+} puffs" was correlated with the generation and propagation of intracellular Ca^{2+} waves in oocytes.

Figure 12 also shows the versatility of the CLSM for imaging dynamic processes in thick tissue. Our research addresses asthma, a common lung disease that is mediated by the hyperactivity of small airway smooth muscle cells (SMCs). Most studies have employed cultured SMCs, but a major criticism of this approach is that the isolation of SMCs radically alters their phenotype and cellular physiology. The loss of SMC contractility is the most common form of function loss. In addition, the SMCs isolated are rarely those of the small airways but more often from the trachea.

We, therefore, developed procedures to cut slices (~75 μm thick) of mouse lung in order to study, *in situ*, the Ca^{2+} signaling that occurs within airway epithelial and smooth muscle cells (SMCs). In a lung slice, the structural relationships between the epithelial cells, the SMCs, the airways, and surrounding alveolar tissue are extremely well maintained. Furthermore, the airway SMCs retain the ability to perform repetitive contractions for at least 1 week (in organ culture). These slices can be loaded with fluorescent dyes for monitoring Ca^{2+} (using AM esters). However, many other cells besides the SMCs take up the dye and, as a result of the slice being several cells thick, out-of-focus fluorescence makes conventional wide-field observation impossible. Using the CLSM, it has been possible to locate and study SMCs. The addition of a variety of drugs [e.g., acetylcholine (ACH)] induces rapid increases in Ca^{2+} followed by Ca^{2+} oscillations. These oscillations can persist for some time but are generally inhibited by the removal of ACH with esterase. The cessation of the oscillation is accompanied by a substantial relaxation of the muscle (Fig. 12). We believe this is the first time Ca^{2+} signaling in airway SMCs *in situ* has been studied.[8]

[6] J. S. Marchant and I. Parker, *EMBO J.* **20,** 65 (2001).
[7] J. S. Marchant and I. Parker, *Br. J. Pharmacol.* **132,** 1396 (2001).
[8] A. Bergner and M. J. Sanderson, *J. Gen. Physiol.* **119,** 187 (2002).

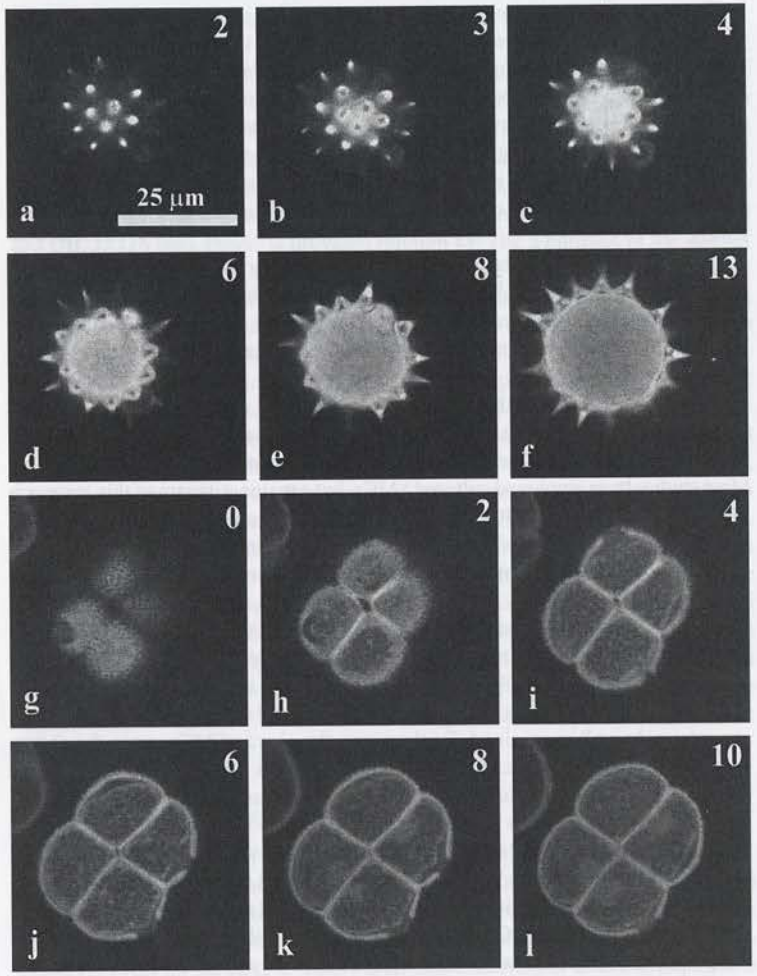


FIG. 11. A series of optical sections through two different pollen grains (a–f and g–l) obtained with the CLSM and the piezoelectrical focusing adapter. The relative depth position of each section (in micrometers) is indicated at the top right of each image. Each image is an average of eight video frames (acquired at 30 fps). Image width and height: 50 μm.

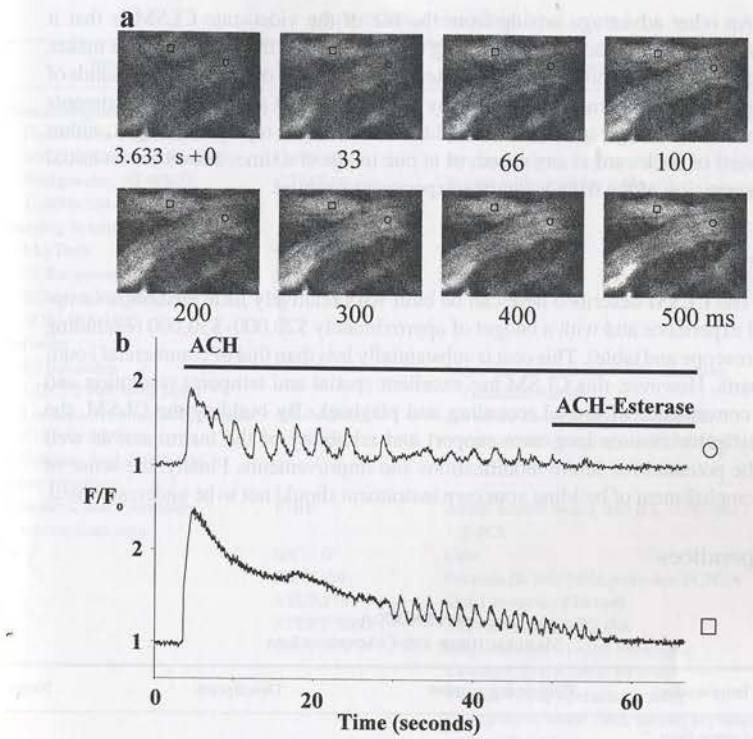


FIG. 12. Confocal imaging of dynamic intracellular Ca^{2+} signals within a slice of lung tissue. (a) A series of selected images recorded from airway smooth muscle cells (SMCs) *in situ* within a lung slice at 30 fps. The acquisition time from the beginning of the sequence is indicated (in milliseconds) below each image. A single frame at each time point is shown. (b) An extended pixel point analysis of the images recorded in (a). The change in fluorescence ratio (F/F_0) was determined from two different cells as indicated by the square and circle. More than 1800 frames were used. These SMCs lie parallel to an airway that is orientated from bottom left to top right. The SMCs were loaded with Oregon Green. Acetylcholine (1 m*M*) was added to the slice over the time range indicated by the bar, inducing a rapid increase in intracellular Ca^{2+} in many SMCs, accompanied by a large, slow contraction. The Ca^{2+} increase occurred within 100 ms and is documented by the first four images in (a). Because the cells are contacting, after ACH application, it was necessary to track the location of the cells so that the same pixel area could be monitored from each individual SMC. Following the initial Ca^{2+} increase, the intracellular Ca^{2+} began to decline and intracellular Ca^{2+} oscillations occurred with a declining baseline and amplitude. The presence of these oscillations could continue for several minutes but the application acetylcholine esterase (85 units/ml; indicated by a bar) inhibited the Ca^{2+} oscillations. This reduction in Ca^{2+} was accompanied by a large relaxation of the SMCs.

An other advantage arising from the use of the video-rate CLSM is that it provides rapid feedback when changing focus or moving the objective. This makes the use of the microscope simple and interactive. The ease of recording thousands of images on reusable media removes any inhibition about performing experiments because of wastage of media. In addition, the instant replay of images, either forward or backward at any speed, or at one image at a time, allows for an initial interpretation of the data during the experimental period.

Summary

The CLSM described here can be built with relatively little electronic or optical experience and with a budget of approximately $20,000–$30,000 (excluding microscope and table). This cost is substantially less than that of commercial counterparts. However, this CLSM has excellent spatial and temporal resolution and the convenience of digital recording and playback. By building the CLSM, the investigator ensures long-term support and reliability of the instrument as well as the potential for future modifications and improvements. Finally, the sense of accomplishment of building your own instrument should not to be underestimated.

Appendices

APPENDIX A
MANUFACTURER AND COMPONENT LIST

Item/vendor	Part/catalog number	Description	Notes
Air-cooled argon laser			
Melles Griot Laser Division	532-A-A04	Ion-laser Head 100-mW argon laser 5956	
2051 Palomar Airport Drive	05224	Cooling fan assembly AC2-10B	
Carslbad, CA	176B-120B	Ion-power supply lab 120 V 4852	
(760)-438-2131			
Antivibration table			
Newport Corp.	VW-3046-opt-021022	Air table, stainless steel top (30 × 46 inches), 1-inch holes centered holes, 0.25, inch, 20 thread	
1791 Deere Ave.			
Irvine, CA 92714			
(714)-863-3144			
Scanning mirror assemblies			
GSI Lumonics	000-30150B	CRS 8-kHz scanner including driver boards, cables, pixel clock, and mirror	
4E Crosby Drive			
Bedford, MA	000-3008001	M3H scanner	
(781)-275-1300	E11-132095	OATS driver board for M3H	
	310-146611	Paddle mirror assembly	
	312-153261	72-inch extension cable	
		Tune OATS driver for 30- to 60-Hz sawtooth waveform	

APPENDIX A (*continued*)

Item/vendor	Part/catalog number	Description	Notes
Photomultiplier			
Hamamatsu Corporation	R3986	Photomultiplier tube (side on)	
360 Foothill Road, Box 6910	C4900-00	Power supply	
Bridgewater, NJ 08807	C7247-01	Socket with amplifier (side on)	
1-(800)-524-0504			
Imaging boards			
Mu-Tech	90-10002-E00	MV-1000-20MHz	
85 Rangeway Road	91-00VC7-001	MVC-7 cable	
Billerica, MA 01862			
(978)-663-2400			
Software			
IO Industries		Video Savant with hard disk recording (standard edition)	1
102-252 Pall Mall Street			
London, ON N6A 5P6, Canada	Available on request	A custom-built compatible computer system with imaging board installed	
519-663-9570			
Contact: Andrew Sharpe			
Basic computer			
Treasure Chest Computers	P3BF	ASUS mother board, 440 BX, APG slot 1, 5 PCI	2
tccomputers.com	EN7237	Case	
	IT277356	Pentium III 800-MHz processor FCPGA	
	ASUA370	Slot 1 to socket 370 card	
	XPERT 2000	ATI video card, APG slot	
	G810	Large-monitor 21-inch ViewSonic	3
		Yamaha CD-RW, 16 × 10 × 40	4
		Windows 2000 operating system	5
		Floppy drive, sound card, mouse, keyboard, network card	6
	32 × 64100S	Recommend 2 × 256 MB of memory (can add up to 1 GB)	7
Hard drives			
DC Drives	A19160	Adaptec PCI Ultra 160 SCSI controller	8
3716 Timber Drive	TN318200LW	Quantum hard drive, 18.2 GB, 10,000 rpm, 160 SCSI (two required)	9
Dickinson, Texas 77539			
1-(800)-786-1160	ST320420A	Seagate, 20 GB/7200 rpm, ATA/66	10
Shutter			
Vincent Associates	LS6T2	Laser shutter	11
1255 University Ave.	122-BP	Open frame shutter driver	12
Rochester, NY	710P	7-Pin cable	
1-(800)-828-6972			
Optical components			
Nikon: See local dealer	84220	CFW ×10 eyepiece (additional to microscope)	13
Omega Optical	XL06	488NB3 argon laser line filter	
P.O. Box 753	XF2037	Dichroic filter 500 DRLP	14
Brattleboro, VT 05302	XC100	Filter cube for Nikon to hold filters	15

continued

APPENDIX A (*continued*)

Item/vendor	Part/catalog number	Description	Notes
Optical components			
www.omegafilters.com	XF3006	18-mm long-pass OG15	
(802)-254-2690	XF22	18-mm excitation filter 485DF22	
	XF22	18-mm band pass 530DF30	
	XF22	Dichroic filter 505DRLP	
Opto-mechanics			16
Coherent Auburn Group	61-1137	Rectangular aperture	
2303 Lindbergh Street	61-3497	12-inch rail (1)	17
Auburn, CA 95602	61-3513	Rail carrier, 1 inch (2)	
1-(800)-343-4912	53-9775	Filter wheel (2)	
	53-2432	Top adjustable mirror mount	
	OG-515	Long-pass glass filter	
Edmund Scientific	K54863	Iris mount, 38 mm	
101 East Gloucester Pike	K53907	Iris, 37-mm OD	
Barrington, NJ 08007	K52557	Polarizer, 42 mm	
(856)-573-6250	K52572	Rotary optic holder	
	K55177	0.25-Inch, 20 thread screws, 0.75 inch long	
OptoSigma	034-2230	Broad brand mirror, 1 inch diameter (4)	
2001 Deere Ave.	112-0250	Mirror mount (3)	
Santa Ana, CA 92705	112-0264	Mirror holder, 1 diameter (3)	
(949)-851-5881	199-0161	Ball driver/4 20 (1)	
	148-0210	Post, 1 inch (~5)	18
	148-0220	Post 1.5 inch (~10)	19
	148-0230	Post 2 inch (~10)	20
	148-0240	Post 3 inch (~5)	21
	148-1310	Post holder 1 inch (~10)	
	148-1320	Post holder 2 inch (~10)	
	147-0443	Post mounting bases (~20)	
	111-0080	Lens holder, fixed	
Microscope			
Nikon: See local dealer	90101	Inverted, Nikon Diaphot 300, with 80/20 and 100/100 light-directing cubes	22
	85006	Objective, ×40 or ×60 oil, NA ~1.3	23
	84220	Objective low power, ×10, aid for aligning laser beam	24
	90131	Epifluorescence system for scanning the specimen	25
	See Omega Optical	Filter cube/holder	26
	See Omega Optical	Filters set for Fluo-3, Ex 485, Dichroic 505, Barrier 510LP	26
	90145	3-Position cube changer cassette	27
	87530/87531	Hg lamp housing and socket	28
	78589	Hg bulb	29
	87505	Condenser lens	30
	79444	Zoom lens	31

APPENDIX A (*continued*)

Item/vendor	Part/catalog number	Description	Notes
Chiu Technical Corp.	MX-75/100R	100-W power supply for Hg or Xn	
252 Indian Head Rd.			
Kings Park, NY 11754			
Polytec PI	P-721.10	Piezoelectric focus with LVDT	
23 Midstate Drive			
Suite 212	E-662.LR	LVPZT amplifier controller	
Auburn, MA 01501			
(508)-832-3456			
Electronics components			
Newark Electronics	99F1014	Enclosure for electronics (control box)	
217 Wilcox Ave.		Valuline HC 14104	
Gaffney, SC 29340			
1-(800)-463-9275			
FLW	HAA5-1.5/OVPA	Power supply ±5 V	
350 Cadillac Ave.	HBB15-1.5A	Power supply ±15 V	
Costa Mesa, CA 92626	HBB24-1.2A	Power supply ±24 V	
www.flw.com			
Digi-Key Corp.		Front panel	32
701 Brooks Ave. South	73JA103-ND	10 kΩ, ten-turn potentiometer (3)	
Thief River Falls, MN 56701	73JA503-ND	50 kΩ, ten-turn potentiometer (1)	
1-(800)-344-4539	412KL-ND	Potentiometer knob, black (4)	
www.digikey.com	CKN1038-ND	4PDT toggle switches (4)	
	67-1147-ND	3-mm red LED (5)	
	67-1148-ND	3-mm green LED (2)	
	RLC010-ND	Mini volt meter (1)	
	381N103-ND	10 kΩ, 1W, 1 turn plastic potentiometer (1)	
	8558K-ND	Knob for potentiometer (1)	
	CKN1021-ND	SPDT switch (1)	
	CKN1035-ND	DPDT switch (2)	
	CKN1121-ND	Push-button switch (1)	
		Back panel	32
	ARFX1064-ND	Panel mount BNC receptacle (8)	
	CP-1250-ND	Panel mount 5-pin receptacle (2)	
	MFR15-ND	Sub D 15-pin connector (1)	
	A2047-ND	Sub D 9-pin connector (1)	
	Q204-ND	Plug receptacle	
		Other	
	VFP-KIT-ND	Shrink tubing kit (various sizes)	
	1602-KIT-ND	Nuts and bolts kit (various sizes for mounting)	
	J216-ND	Stand-off threaded spacer for circuit boards	
	J212-ND	Stand-off threaded spacer for circuit boards	
	3122K-ND	Isolating nylon washers, no. 6 screw	
	F-KIT-ND	Various capacitors	
	PHD1-KIT-ND	Various ceramic capacitors	
		Hook-up wire	

continued

APPENDIX A (*continued*)

Item/vendor	Part/catalog number	Description	Notes
		Cables	
	CP1050-ND	In-line plug, 5 pin (4) (CRS and PMT cable)	
	MMR09-ND	In-line D plug, 9 pin (shutter cable)	
	A120-100-ND	Multiconductor cable to make cables	
	A3213-100-ND	4-Conductor cable	
		Chips	
	296-1626-5-ND	Quad, 2 input, NAND gate, SN74LS00 (1)	
	296-2118-5-ND	12-Bit binary counter, CD74HCT4040E (1)	
	CD4070BCN-ND	Quad, 2-input exclusive OR gate (1) Replacement part for HCC4070	
	LT1012-CN8-ND	Op amp LT1012 (1)	
	AE9808-ND	IC socket, 8 pin, gold (for op amp)	
	AE9814-ND	IC socket 14 pin, gold (for NAND gate)	
	AE9816-ND	IC socket 16 pin, gold (for binary counter)	
	AE9820-ND	IC socket 20 pin, gold (for AD converter)	
	Analog Devices	10-Bit ADC	
		Connectors for M3H boards	
	109M-ND	DB-9 connector, male for ramp and position	
	WM3702-ND	6-Pin power connector housing—Mini Fit	
	WM2501-ND	Pins for above	
	M7PXK-1506R-ND	D subcable, single end male, pin	
		Connector for CRS board	
	M1AXK-1636R-ND	Single-ended socket connector/cable for J3 (power)	
	M1AXA-2436R-ND	Single end socket/cable for J1 (data)	
Radio Shack: local stores in United States	2760168	PC boards	
		Various resistors	
	2750602	SPST power switch (1)	
Analog Devices 3 Technology Way P. O. Box 9106 Norwood, MA 02062 (781)-329-4700	AD7393AN	10-Bit ADC	
L-Com 45 Beechwood Drive North Andover, MA 01845 (978)-682-6936	CC174-10 CSM15MF-10 CSM25MF-10	Coaxial cable, 10 ft, BNC-BNC (8) MF15 pin parallel cable, pin to pin (1) MF25 pin parallel cable (1)	
Miscellaneous			
Carolina Biological Supply P.O. Box 6010 Burlington, NC 27216 1-(800)-334-5551	30-4264	Slide of mixed pollen grains	

APPENDIX A (*continued*)

1. When purchasing, request the drivers for Mu-Tech board that have the ability to perform (a) pixel reversal and (b) image distortion correction, (c) live display during recording, and (d) advanced recording as developed for Dr. Sanderson. An evaluation version of the software is available at their Web site.
2. This mother board works with the Mu-Tech card and Video Savant. Other mother boards may not. Contact IO Industries for other compatible mother boards. It is not worth buying the latest thing.

3–7. The specifications of these computer components frequently change with technology advances. A review of each component is recommended at the time of purchase for the current model.

4. A read–write CD provides the option of archiving and back-up of data. The RW-CD-ROM is rapidly being replaced by RW-DVD (ROM or RAM).
6. No specific requirements for the basic components of the computer.
8. A PCI controller card for the SCSI drives is preferable to a controller built into the mother board, as this allows for upgrades in disk access speed without changing the whole system.
9. These SCSI drives have probably been superseded by newer drives that have a larger capacity, faster access speed, and are cheaper.
10. The Seagate hard disk is for the operating system and can be anything. A large capacity and fast access time are recommended.
11. The LS6T2 was used because it has a wider aperture, making alignment less critical. However, this shutter cannot open and close as fast as the 3-mm-diameter shutter. The Teflon-coated blades are not usually used for high-powered lasers, but these seem to be no problem in our systems.
12. The power supply/controller for the shutter is a simple way to integrate the shutter into the control box.
13. This eyepiece needs to be machined and attached to the rectangular aperture such that the knife edge shutters are at the focal plane of the eye piece.
14. The size and shape of filter will depend on mounting: Square or circular.
15. This can be custom made by a machine shop or substituted with a filter wheel.
16. All optical mounts and components can be bought from any optics company.
17. Rail is cut into two parts to use for sliding mount of eyepiece and scanner mirrors.

18–21. The exact number of posts at set heights that are required is difficult to predict because this will depend on the alignment of the system. Having enough on hand to swap components avoids considerable frustration.

22–31. Original Nikon Diaphot 200/300 parts are no longer in production. A substitute microscope may have to be purchased.

26. A filter cube cassette allows the filter set to be easily and quickly inserted into or removed from the illumination pathway.
32. All electrical components can be purchased from any vendor. It is recommended that the catalog number is checked before purchasing.

APPENDIX B
LAYOUT OF FRONT PANEL CONTROLS[a]

Item	Indicator/control	Function	Part number
1	Mini volt meter	Indicator of laser output power (mW)	RLC010-ND
2	10 kΩ potentiometer knob	Control of laser output power	381N103-ND/8558K-ND
3	Switch	Placement of laser in stand-by mode	CKN1021-ND
4	Green LED	Indicator of laser status	67-1148-ND
5	Red LED	Indicator of main power	67-1147-ND
6	Switch	Main power switch	RS 2750602
7	10-Turn, 50 kΩ potentiometer	PMT gain control	73JA503-ND/412KL-ND
8	10-Turn, 10 kΩ potentiometer	Image width, CRS scan control	73JA103-ND/412KL-ND
9	10-Turn, 10 kΩ potentiometer	Image height, M3H scan control	73JA103-ND/412KL-ND
10	10-Turn, 10 kΩ potentiometer	Vertical image position, M3H scan control	73JA103-ND/412KL-ND
11	Green LED	Shutter open indicator	67-1148-ND
12	Switch	Shutter open/closed, trigger control	CKN 1035-ND
13	Push button	Trigger to record single frame	CKN1121-ND
14	Red LED	Indicator of power to circuits	67-1147-ND
15	Switch	Switch for power to circuits	CKN1038-ND
16	Red LED	Indicator of power to PMT	67-1147-ND
17	Switch	Power switch for PMT	CKN1038-ND
18	Red LED	Indicator of power to CRS	67-1147-ND
19	Switch	Power switch for CRS	CKN1038-ND
20	Red LED	Indicator for power to M3H	67-1147-ND
21	Switch	Power switch for M3H	CKN1038-ND
22	Switch	Select 30/60 Hz	CKN1035-ND

[a] Part numbers are included in Appendix A. See Fig. 10 for design.

APPENDIX C
LAYOUT OF BACK PANEL CONNECTIONS[a]

Item	Connector	Function	Part number
1	Female BNC	In PMT luminance	ARFX1064-ND
2	Female BNC	Out PMT luminance	ARFX1064-ND
3	Female 5 pin	High voltage and power to PMT	CP-1250-ND
4	Female Sub D 25 pin	Built-in to argon laser control card	Laser part
5	Female BNC	Not used	ARFX1064-ND
6	Female BNC	Parallel port trigger out	ARFX1064-ND
7	Female BNC	Not used	ARFX1064-ND
8	Female BNC	V sync out	ARFX1064-ND
9	Female BNC	H sync out	ARFX1064-ND
10	Female BNC	Shutter trigger in	ARFX1064-ND
11	Female 5 pin	Power and control connector to CRS	CP-1250-ND
12	Female sub D 15 pin	Power and control to M3H scanner	MFR15-ND
13	Female sub D 9 pin	Shutter control	A2047-ND
14	Plug receptacle	Main power	Q204-ND

[a] Part numbers are included in Appendix A. See Fig. 10 for design.