

# High-speed CCD movie camera with random pixel selection, for neurobiology research

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## ABSTRACT

We have designed and built a CCD camera capable of producing movies at over 1000 frames per second. For maximum frame rate, we have incorporated the ability to digitize only the pixels of interest, user-selectable from a custom LabView interface. With a resolution of 64x64 pixels, the system is intended to bridge the gap between fast, small photodiode arrays, and slow, high-resolution scientific CCD cameras. The system was designed for imaging neurons labeled with voltage-sensitive dyes, to allow the simultaneous recording of neural activity in a large number of neurons, or to probe the membrane voltage at many different points of a single neuron.

**Keywords:** Potentiometric probe, voltage-sensitive dye, optical recording, high-speed programmable CCD, neural imaging.

## 1. INTRODUCTION

The brain is probably the most complex structure we know of, with on the order of a trillion neurons, each with hundreds or thousands of connections (synapses) to other neurons. There are major gaps in our understanding of how a structure that fits inside a skull and consumes only about 100 watts can have huge memory capacity and very rapid recall. This amazing organ effortlessly provides creativity, emotions, language, and many other brain activities that vex artificial intelligence researchers trying to emulate them with digital computers. We hope that with further study of living neural systems, we will begin to unravel the mysteries of neural information processing, and perhaps we can borrow some clever ideas from Mother Nature in the design of artificial computing systems.

There is increasing evidence that much of neural information processing is a result of the collective activity of large ensembles of neurons (e.g., Cochin *et al.*, 1994; Wu *et al.*, 1994). Neural cell cultures provide a convenient and accessible system to study this activity. Microelectrodes are the standard tool of neurophysiology. They allow one to record signals from only a few cells at a time, since each electrode requires a micromanipulator, and impaling the cells is a tedious and invasive procedure. For over two decades (Davila *et al.*, 1973), it has been possible to record neural activity optically, by monitoring the fluorescence (or absorption) of neurons stained with voltage-sensitive dyes. These potentiometric probes sense the voltage across the cell membrane, which changes from about -60 mV to about +40 mV during an action potential (the signal that causes neurotransmitters to be released at the synapse). By shifting the charge on a dye molecule in the membrane, the electric field changes the optical properties of the dye (Loew, 1993). This process occurs much faster than the voltage transient of an action potential, which is typically a couple milliseconds in vertebrate neurons. Unfortunately, the change in fluorescence during an action potential is only a couple percent of the resting fluorescence level, per 100 mV change in membrane potential. This, and the rapidity with which neural signals come and go, has made optical recording of neural activity a daunting task.

Until recently, only photodiodes have proven to be sensitive and fast enough for imaging of voltage-sensitive dyes. Most studies to date have used 10x10 or 12x12 monolithic photodiode arrays, although arrays of discrete diodes have also been used (Chien and Pine, 1991). Systems with larger photodiode arrays are quite

expensive. Inexpensive charge-coupled device (CCD) imagers have greatly expanded our capabilities for scientific imaging. These offer high quantum-efficiency, low noise, excellent linearity, and with new amplifier and A/L) conversion technology, high-speed readout of the CCD.

We created a relatively inexpensive high-speed CCD (HSCCD) movie camera with a resolution greater than that of commonly used photodiode arrays, for imaging the dynamics of neural activity at speeds substantially faster than CCDs commonly used for biological microscopy. We accomplished readout of more than 1000 frames per second (fps) by incorporating a novel digitizing scheme, in which only pixels of interest are digitized.

## 2. HSCCD SYSTEM

Our high-speed CCD camera system (Figs. 1 & 2) consists of a headboard mounted on the side port of a fluorescence microscope, a rack of electronics for controlling the camera and amplifying the image pixel signals, a Macintosh computer with interface boards (more below), and a LabView user interface for setting camera operating parameters and displaying image data. Also connected to the microscope is an inexpensive CCD camera (Sony SSC-M350) for taking still phase-contrast images of neural networks at 640x480 pixel resolution. The total system, excluding the microscope, cost about \$20K.

### 2.1 CCD chip

We designed our camera around the Tektronix TK064 CCD chip, a 64x64 pixel array- This CCD has 27 urn square pixels with 500,000 electron full-well capacity (1.7 mm square imaging area). It is backside-illuminated and anti-reflection coated for a quantum efficiency >50% in the visible. It has two amplifiers, for readout in either direction (only one is used) with a 0.5 uV/electron sensitivity and a specified readout noise of 15 electrons at room temperature. It employs 3-phase clocking, with a 4th phase for on-chip binning of photoelectrons from adjacent pixels. The serial register pixels have twice the full-well capacity of the image pixels. The chip's small size (and thus low parallel phase capacitance of 55 pF) makes it ideal for high-speed readout of pixels.

### 2.2 Computer system

Our now-antique Macintosh IIfx, running LabView 3.0 (National Instruments), controls the camera electronics via a digital interface card (National Instruments, NB DIO-32F). This has 35 two-way TTL channels, of which 20 are used. The pixel data signal is digitized by an analog-to-digital card (National Instruments NB A2000) at the maximum conversion rate of 1 Mpixel/sec. It is a 12-bit, track-and-hold A/D converter with a +5V input range, triggered externally by the CCD electronics. We also use an 8-bit frame grabber (Quick Capture, Data Translation) for still images from the Sony CCD. The computer has 20 MB of RAM, enough for a movie length of about 500 full frames, or several seconds of subregion images at 1000 fps (frames/sec).

(soma) and larger neurites of a single neuron, or image the cell bodies {somata} of a number of neurons in a cultured neural network (Figs. 5 & 6).

Our CCD control software also includes the ability to carry out on-chip binning of rectangular groups of pixels to increase the signal when imaging dim specimens (Fig. 6c). This also speeds up frame readout, since there are fewer pixels to digitize. The software control panel (Fig. 3, top) allows the user to adjust the integration time to optimize signal level and frame rate.

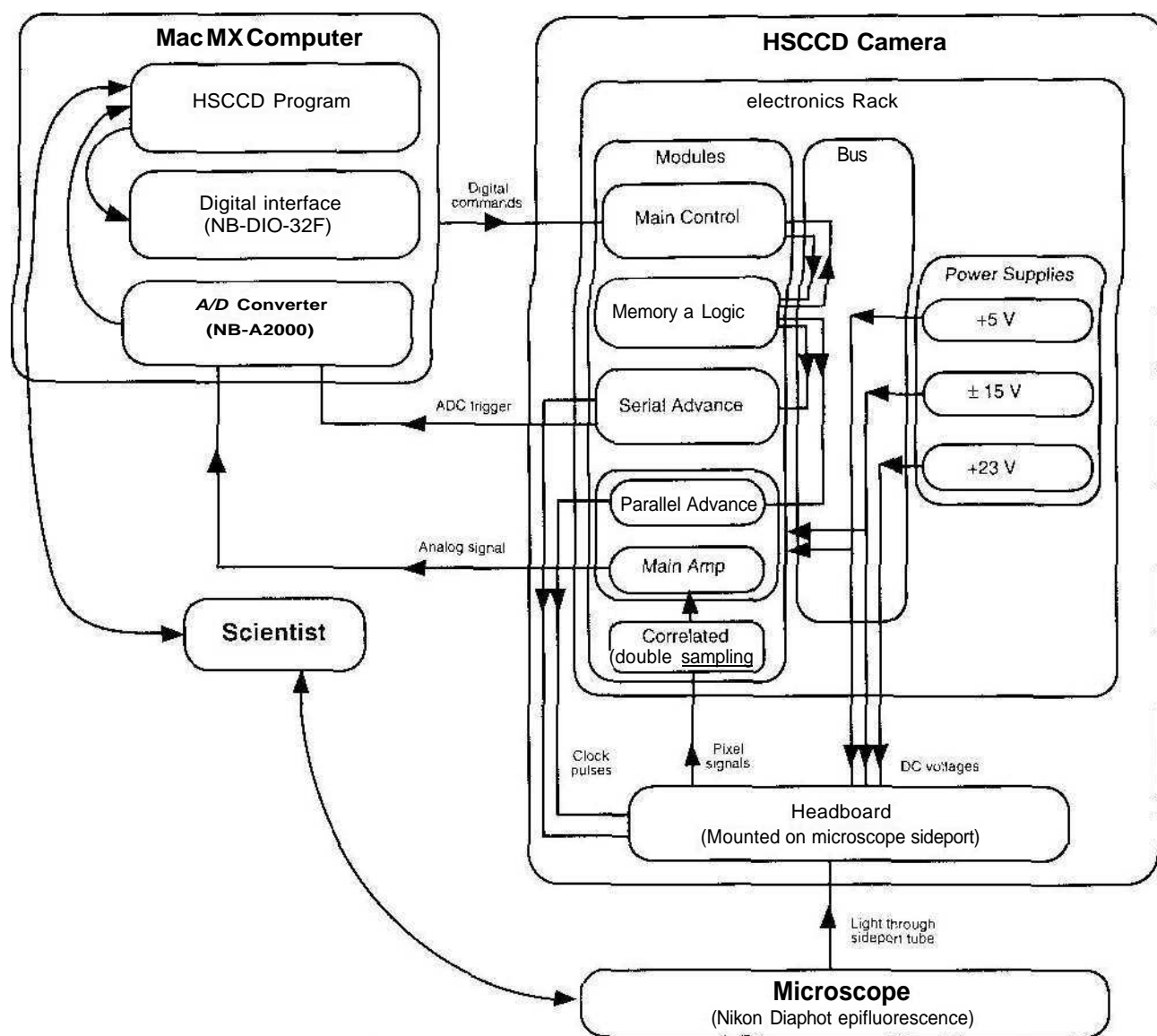


Fig. 2. High-speed CCD system diagram.

## 2.4 HSCCD electronics

The rack of camera electronics (Fig. 1) contains regulated power supplies and five modules: Main control, Memory and Logic, Serial Advance, Parallel Advance, and Main Amplifier.

The Main Control module takes input from the digital interface card in the computer, and has several test features for manual operation. It contains the master clock, a 10 MHz crystal oscillator, integration timer, and frame counter.

The Memory and Logic module contains an 8K SRAM chip (Micron Technology MT5C6408) that is programmed by the computer before a movie is taken. Each pixel on the CCD chip is associated with a word in memory, containing a command telling the electronics what to do with that pixel. The bits 0-5 stand for (0) Serial Advance, (1) Serial Binning, (2) Digitize, (3) Parallel Advance, (4) End of Frame, and (5) Integrate. Bits 6 and 7 are not used. There is a memory address counter that is incremented in sync with the clocking of the pixels. The output of the memory, the current pixel command, is interpreted by a TTL circuit that controls the other modules. For example, the pixels of interest all have bit 2 set high, which pauses the clock for one microsecond and triggers the A/D converter, and the last pixel of each row has bit 3 set high, which causes a parallel advance.

The Serial Advance and Parallel Advance modules contain pulse shapers, adjustable pulse height power supplies, and output buffers that send the 3-phase pulses to the headboard. We use 50 ns serial pulses (FWHM, 15 ns rise and fall times, -5 to +5 V), and 100 ns parallel pulses (FWHM, 15 ns rise and fall times, -8 to +2.3 V). There is a fourth serial phase that is held high when binning columns. Otherwise, it is clocked as if it were phase 3. Binning of rows is done by executing more than one parallel advance without reading out the serial register.

The Main Amplifier contains filters and a two-stage low-noise high-speed amplifier. Gains from 80x to 1280x can be selected with a front panel switch.

The headboard contains the CCD chip itself, cooled by a Peltier thermoelectric device, amplifiers for the serial and parallel phases, and a low-noise preamplifier for the pixel signals.

## 2.5 Mechanical and optics

The headboard is mounted in a light-tight box with micrometer screws for precision CCD chip alignment. The microscope image is focused on the CCD chip with a Fujinon video zoom lens (F 1.2, 12.5 to 75 mm) with +4 close-up lens kit, and an AR-coated transport lens. The zoom can be adjusted to match the specimen size to the CCD imaging area, with no vignetting or distortion.

We use a Nikon Diaphot inverted epifluorescence microscope, with phase contrast optics. The output of the 100 W mercury lamp is stabilized by an optical feedback system (Chien and Pine, 1991). A fiber optic placed in the illumination path carries light to a photodiode, which controls a current shunt that modulates the power to the lamp to keep intensity fluctuations below 0.1% RMS. We use a Nikon rhodamine filter cube, and Nikon Fluor 20x/0.75 numerical aperture and 40x/0.85 numerical aperture objective lenses. The microscope is set up with standard electrophysiology equipment to allow comparison of optical signals with those recorded by conventional microelectrodes. The electrophysiology, the camera, and the illumination shutter are all triggered by the computer at the click of a mouse.

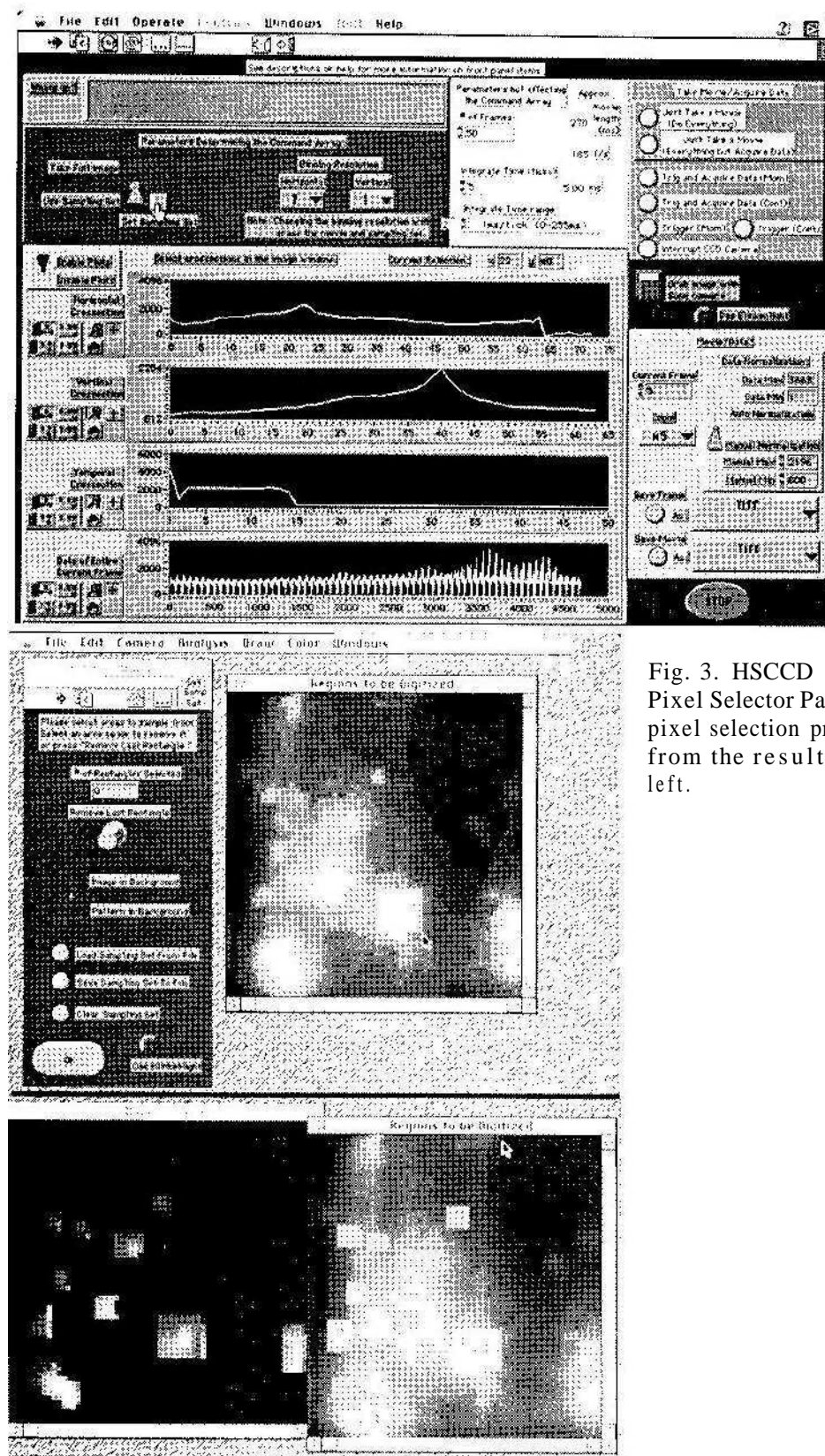


Fig. 3. HSCCD Main Control Panel (top) and Pixel Selector Panel (bottom). An example of the pixel selection process is presented, with a frame from the resulting high-speed movie at bottom left.

### 3. SYSTEM PERFORMANCE

#### 3.1 Movie speed

The HS CCD takes 400  $\mu$ s to clock out a full frame, without digitizing any pixels. Since each pixel that is digitized pauses pixel readout for 1  $\mu$ s, the frame rate is a function of the number of pixels selected, as well as the integration time selected. Thus, when the integration time is set to half of the readout time, we can make movies at >1000 fps for 200 pixels or fewer, as shown in Fig. 4. Vertical smearing from light collected during frame readout is usually tolerable with this scheme.

The software currently instructs the camera to clock each pixel in the CCD array out through the serial register, at either 10 MHz or 1 MHz, depending on whether it is being digitized or not. We plan to attain further speedup by executing just parallel advances (no serial advances) in situations where groups of adjacent rows have no selected pixels. The last unwanted row will be fast-clocked through the serial register, so the serial register wells will be empty and ready to accept charge from the next row, which contains selected pixels. This scheme will save approximately 6.4  $\mu$ s per unselected row (dashed curve, Fig. 4). Thus, if one selected a 20x20 region, 43 rows would be fast erased. This saves 275  $\mu$ s per frame, boosting the frame rate, from 770 to 1130 fps, if the integration time equals half the readout time. Our pixel command array memory allows us to try out new clocking schemes easily, just by altering the LabView program. No hardware changes are necessary.

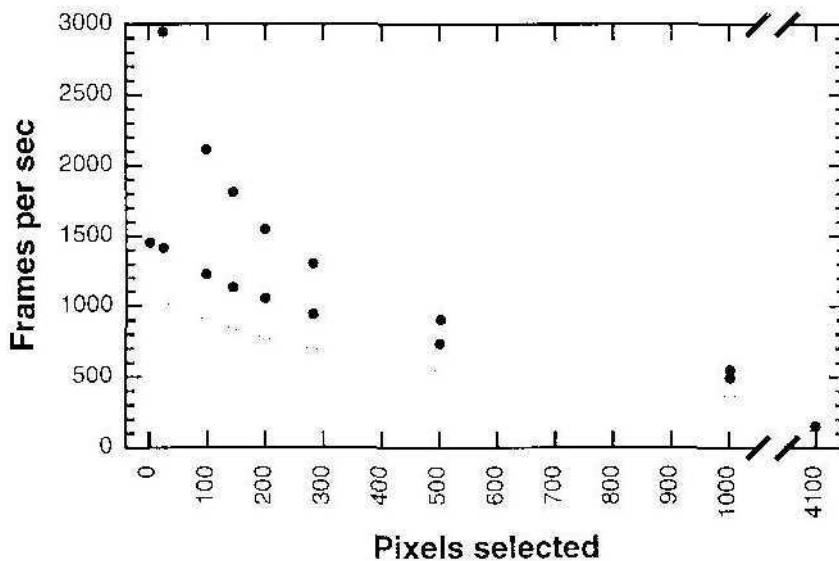
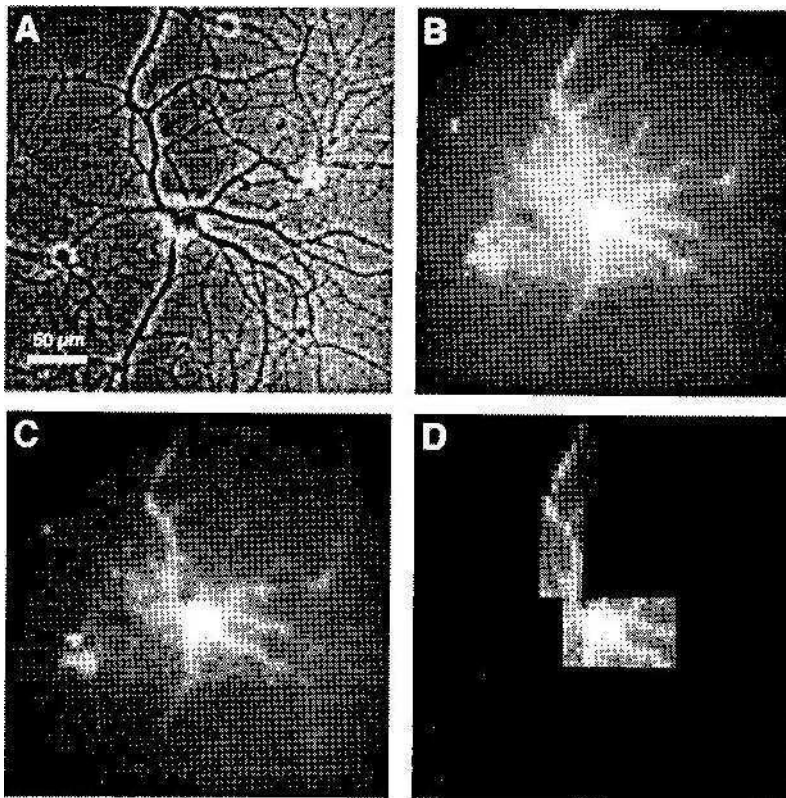


Fig. 4. Frame rates were measured with an oscilloscope, for a variety of pixel selections, using integration times equal to the frame readout time (open circles), or equal to half the frame readout time (filled circles). Top curve (dashed line) is the calculated best-case readout rate, for a single square region selected, after implementation of parallel fast erasing of unwanted rows (see text).

#### 3.2 Sensitivity and noise

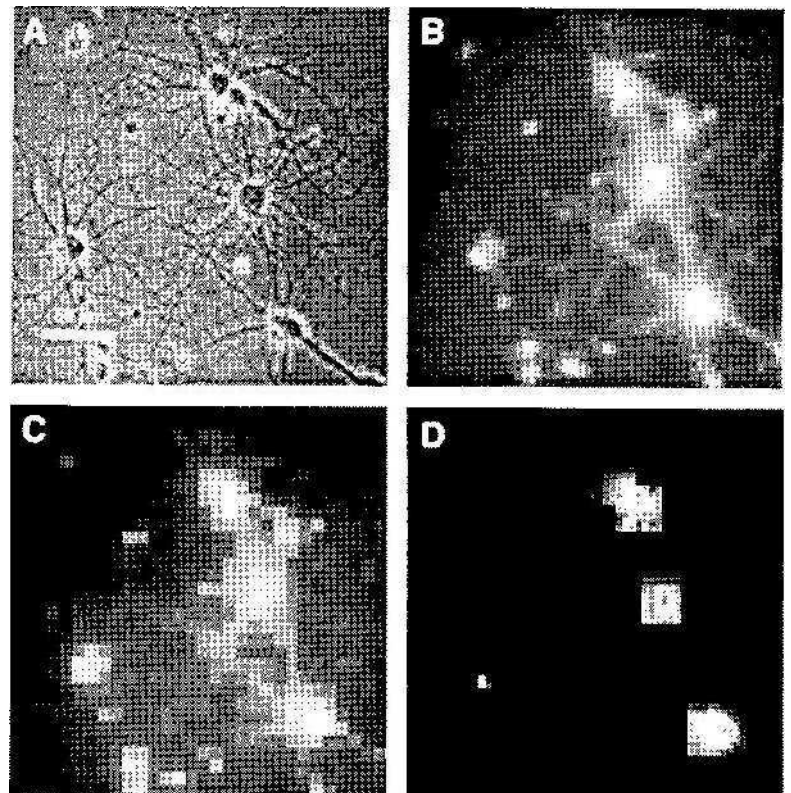
For a typical 1000 fps movie of cultured hippocampal neurons stained with RH237 (Molecular Probes), we integrate for 300  $\mu$ s/frame (Fig 6d)). Using the 20x/0.75 NA microscope objective and the full intensity of the mercury lamp, we get approximately 20,000 photoelectrons/pixel/frame from the cell soma pixels. The complete image of the neuron's soma (from which voltage sensitive dye signals are traditionally obtained) provides about 300,000 photoelectrons/frame. The shot noise from this number of photoelectrons is about 550 electrons, or about 0.2%. This will allow detection of fluorescence changes of 1% or more with a good signal-to-noise ratio. Unlike with slow-scan CCDs that use long (several second) integration times, dark signal is negligible for us. From the CCD specifications, dark current noise for a 300  $\mu$ s integration time is expected to be only 5 electrons. We are in the process of implementing a noise-reduction circuit that is a variation of correlated double-sampling. We estimate the total noise through all the electronics will be 16 electrons rms

(referred to the CCD input, 1 MHz bandwidth). Thus, for the above example, and even for far fewer photoelectrons, the photon shot noise will be the dominant noise source.



**Fig. 5.** Embryonic rat hippocampus neuron, cultured for one month, stained with voltage-sensitive dye R11237. **A.** Phase contrast image from video camera. **B.** Full-frame HSCCD fluorescence image, with 10 ms integration time ( $\sim 70$  fps), with 0.6 neutral density excitation filter. **C.** Same image, after resizing with a bicubic spline interpolation in Photoshop, showing details not apparent in the more pixelated original. **D.** Subregions covering the cell soma and a major dendrite, imaged at  $\sim 900$  fps, with 300  $\mu$ s integration time, at full lamp intensity.

**Fig. 6.** Neural network of embryonic rat hippocampus neurons, after 1 week in culture, stained with RH237. **A.** Phase contrast image from video camera. **B.** Full-frame fluorescence HSCCD image, with 15 ms integration time ( $\sim 50$  fps), with 1.6 neutral density excitation filter. **C.** Same field of neurons imaged with  $2 \times 2$  binning, which reduces readout time to one ms, and boosts the signal level by a factor of four. A surprising amount of image detail is retained, even at  $32 \times 32$ , which can be noted by viewing the image from a distance. **D.** Subregions covering four neuron somata were imaged with no binning at 1000 fps (300  $\mu$ s integration time), using full lamp intensity.



## 4. DISCUSSION

### 4.1 Related systems

A few groups have conducted voltage-sensitive dye recordings using commercially available CCD cameras. The Photometrics CH220 is a computer controlled, cooled 12-bit CCD camera that has been sped up with a custom command set (Lasser-Ross *et al.*, 1991) to allow imaging of an 18x18 pixel region at 100 fps, or a 50x50 pixel region at 40 fps. When used with the intracellularly-injected potentiometric dye JPW1114, it was necessary to average 16 trials to observe clear optical signals at 50 fps (Kogan *et al.*, 1995). The same camera was used to image activity in slug procerebral lobe labeled with Di-4-ANEPPS (Kleinfeld *et al.*, 1994), at 9 fps (full-frame) or 27 fps for a 30x100 pixel binning scheme. The Cohu 6510 CCD has been used to image activity waves in cardiac tissue labeled with Di-4-ANFPPS at 60 fps, full-frame. (Cabo *et al.*, 1994). An 8-bit frame-grabber was used in this study, providing sufficient dynamic range to observe the relatively large (3%) change in fluorescence of activated cardiac tissue. None of those systems permit the selection of arbitrary groups of pixels as with our system, and attain their modest speedup by binning of rectangular regions,

Slow-scan CCD cameras (several-second frame readout) have also been used in voltage-imaging experiments that discarded temporal information but showed differences between stimulated and non-stimulated trials (Yae *et al.*, "1992; Ioew, 1993; Gogan *et al.*, 1995) . This is equivalent to taking a two-frame movie, which would miss many of the interesting dynamics of neural ensembles.

Laser-scanning systems for voltage-sensitive dye recording have been developed by two groups (Morad *et al.*, 1986; Saggau, 1994). These employ acousto-optic deflectors to rapidly steer a laser excitation spot to user-selectable regions in the specimen. Fluorescence is detected by a single photodiode. The first system can record signals from 128 sites on cardiac muscle labeled with potentiometric dye VVW781 (in a 128x128 grid) every 4 ms, or 250 fps (Morad *et al.*, 1986). The second system employs a reference photodiode in addition to the signal photodiode., to reduce noise due to laser fluctuations (Saggau, 1994). This system can record from, up to 100 user-selectable spots (20 um diam. with a 20x objective) at a maximum of 2000 fps. Saggau demonstrated fast optical signals evoked in a guinea pig hippocampal slice labeled with potentiometric dye RH-414 that matched extracellular field recordings. The main disadvantage of this approach is the cost and technical difficulty of setting up a fast, low-noise laser-scanning system.

A large monolithic array camera (128x128) has been developed by Ichikawa et al, (Ichikawa *et al.*, 1993) that can image full-field at 1666 fps. This has successfully imaged waves of neural activity in rat hippocampal slices labeled with fluorescent potentiometric dye KH-795 and absorption potentiometric dye RI1-155(Iijima *et al.*, 1996). To our knowledge, it has not been used for imaging activity in cultures of dissociated neurons.

### 4.2 Future improvements

We look forward to an improvement in the readout noise level of our system when our correlated double-sampling amplifier is complete. This should give us the signal-to-noise ratio necessary to see small voltage-induced changes in fluorescence in a single trial. Then, spontaneous electrical activity of a cultured neural network could be observed. Spontaneous activity is likely to be more physiologically relevant than artificially evoked activity.

A future version of the camera might employ an additional RAM chip to hold a digitized background image, to be converted back to analog and subtracted from each image of the movie, pixel by pixel, before A/D conversion. This would subtract the large background fluorescence of voltage-sensitive dyes, so that more of the A/D converter's dynamic range could be devoted to the small changes of interest. Such a scheme has been successfully implemented in the 128x128 pixel camera described above (Ichikawa *et al.*, 1993).



The software that controls our HSCCD is always being improved. We will write routines for automated analysis of movies, including averaging of trials, background subtraction, and dye bleaching correction, and digital summing of groups of pixels. We also wish to overlay fluorescence movies on the high-resolution phase-contrast stills, as done by Senseman, et al. (Senseman *et al.*, 1990).

### 4.3 We need better potentiometric probes

The calcium ion is an important intracellular signal, and the invention of calcium-sensitive fluorescent dyes has created a booming field of calcium imaging, that hopes to gain insight into the inner workings of neurons and other physiologically active cells. There are conferences and journals devoted to calcium imaging, yet none for the imaging of voltage-sensitive dyes. This is because calcium signals are generally 10-100 times slower than voltage signals, and calcium-sensitive *dyes* undergo a large change in fluorescence upon binding calcium (an order of magnitude larger than voltage-sensitive dye changes). Thus, standard video-rate cameras can be used for calcium imaging. New high-speed imaging technologies will do much to advance the field of voltage imaging. But we probably will not catch up with the calcium imaging field until more sensitive indicators of membrane potential are developed. One promising new technique employs fluorescence resonance energy transfer (FRET) between two different fluorophores (Gonzalez and Tsien, 1995). Applying the FRET approach to naturally fluorescent proteins, such as Green Fluorescent Protein and its variants, might provide us with cells that come with their own sensitive potentiometric probes built into their membrane.

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