Quantitative Autoradiography Using a Personal Computer

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A video digitizing system based on an Apple II personal computer has been developed for use in quantitative autoradiography. The selection process of an acceptable video camera, an illumination system, and the functions provided in the software are discussed. This system is in routine use and has been duplicated by several other laboratories.

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Autoradiography is an important tool in investigating biologic processes used to determine the distribution of radiolabeled compounds in tissue sections. These tracer distributions can yield information on perfusion, cell energetics, and density of receptor sites. An essential part of such techniques is the use of quantitative optical densitometry to measure the concentration of tracers as represented by film density in the autoradiograph (*1–3*).

The first efforts in quantitative autoradiography (QAR) used point densitometry to measure density, point by point, manually. The primary problem with this technique was operator fatigue. A major advance was the use of a linear densitometer that allowed computer acquisition of the density values along a line, or profile, across the autoradiograph. The disadvantage of this system was the effort required for multiple profiles. The most sophisticated (and expensive) technique developed for QAR is the rotating drum scanning densitometer interfaced to a minicomputer (*4–10*).

More recently, several systems that digitize films using video equipment have appeared (*11–14*). In this paper, we report the development and use of such a system. The basic goal of the hardware development was to provide an inexpensive system that could digitize an autoradiograph and provide density information either on a pixel by pixel basis, as a profile, or as a region of interest (ROI) over several pixels. While the hardware development is largely specific to the Apple II personal computer, the techniques and software that we have developed are more general. The current system is in routine use of QAR studies and has been duplicated for use in other laboratories at our institution. The configuration consists of an Apple II+ computer with 64 kbytes of memory, a Javelin model JE2062 solid state video camera, a Fujinon model C6×17.5–105 mm zoom lens with extension tubes, a Microworks model DS65 video digitizer, and a C. Itoh model 8510 printer.

DIGITIZING HARDWARE

Within our research laboratory, many applications have been developed for the Apple II computer. Given our previous experience and the alternatives available when the project began, we chose the Apple II as the basic system. In order to reduce hardware costs and provide maximum flexibility (variable magnification and random access to any area of the image) we selected video technology for data acquisition.

In 1981, when the project began, the only commercial videodigitizer available for the Apple II that accepted standard NTSC composite video was the DS65 system (Microworks). The DS65 is contained on a single interface card that plugs into an I/O expansion slot in the Apple II and provides a 256 × 256 image with 64 levels of gray. It consists of a video sync detector, a successive approximation analog to digital converter (ADC) and control/data registers (Fig. 1). The DS65 also includes analog adjustments for redefinition of the portion of the video signal intensity that will be digitized. In effect, the adjustments for brightness and contrast are a window, allowing the density range of interest to be spread over the 64 gray levels available.

The sync detectors provide the method of keeping track of the current x-y pixel address in the video frame. The vertical sync detector starts a counter that indicates which of 256 lines of video data is being received. The horizontal sync pulse triggers a second counter that counts up to 256 pixels as defined by a variable oscillator. The setting of the oscillator determines the width of the digitizing matrix. The address of the pixel to be digitized is loaded into two control registers by

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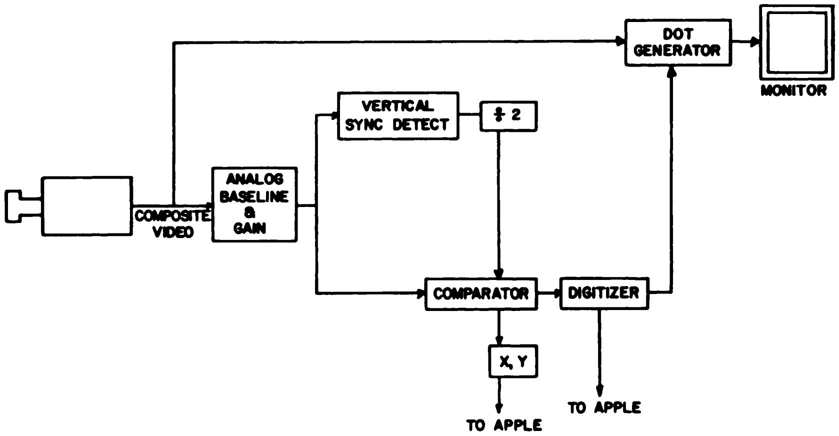


FIGURE 1
Block diagram of DS65 video digitizer for Apple II computer

software. When the counter value matches the register value, a sample and hold circuit samples the video signal for 40 nsec and stretches that value for conversion by the ADC. The ADC provides 6 bits of data (or 64 levels of gray) and requires 12 μsec to digitize the signal. The digitized value is then loaded into a data register and a "done flag" is set within the control register. Since the system is a point by point system, "fast" sampling requires close attention to timing. The assembly level language routine for control of the interface must be designed with care to allow multiple samples per video frame without loading the x-y registers with a value too close to the current horizontal counter values. If the registers are loaded as the desired value "passes by," incorrect sampling may occur.

Three modifications have been made to the DS65 interface. First, the analog one-turn potentiometers for brightness, contrast, and horizontal width were removed and ten-turn potentiometers mounted in a separate box. Second, a circuit was added to allow software selection of triggering the counters on every vertical sync pulse or to skip a frame between triggers. This option was added to allow compatibility with video devices such as video cassette records that use interlaced displays providing 512 video lines by using interleaved 256 line video frames offset by one vertical line.

A third modification was necessary in two of the four DS65 interfaces with which we have had experience. The problem was an artificial increase in the measured density for horizontal positions which were multiples of 16. This was caused by a change in the sampling time of the video signal by an apparent timing error between two 4-bit comparators used in the ADC circuit. By reducing the sampling time slightly with the addition of a one-shot multivibrator, this problem was corrected. While this was specific to the DS65 interface, such problems can be present in any video digitizer and considerable care should be taken in characterizing the behavior of such devices.

FILM ILLUMINATION AND VIDEO CAMERA

The initial effort used a standard fluorescent light box to illuminate the autoradiograph. However, the uniformity of illumination was not constant in time and a cyclic variation was seen when digitizing images, particularly since the time to digitize an entire image was seven seconds. As a result, the configuration shown in Fig. 2 was adopted using four 100 W incandescent lamps with two diffusing screens to present a uniform illumination to the autoradiograph. Since only a single tissue section is digitized at a time, the uniform zone needs to be only 4 to 5 cm on a side. A lens hood reduced the effect of light scatter on the digitized image.

The selection of a video camera and associated lens proved to be the most critical aspect of the system. Initially, we used an inexpensive black and white camera using a low-performance videcon tube. One problem with this camera was the

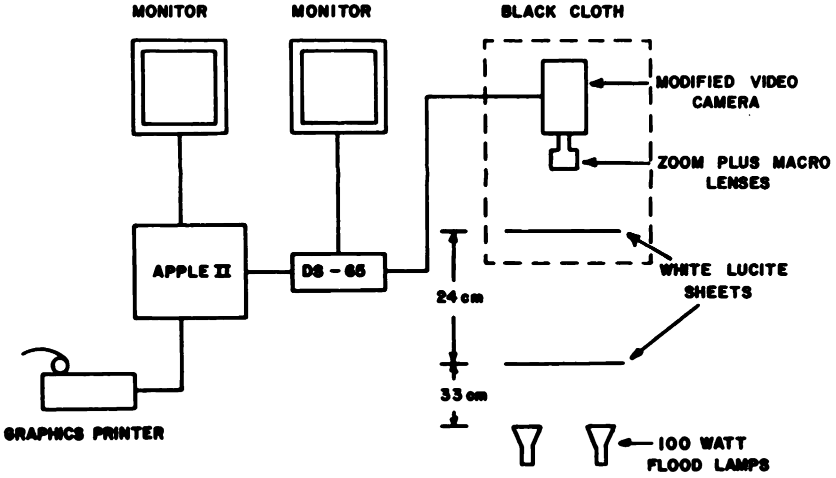


FIGURE 2
Video digitizer system. Tungsten filament lamps and white lucite sheets were used to provide uniform illumination of video camera field-of-view

automatic gain circuit designed to maintain a constant current source to the videcon tube target. The result was a change in video gain as the size and density of the image changed. For example, major errors would occur when using "gray levels" from standards to calibrate an autoradiograph, in some instances producing errors of up to 50%. Disabling the automatic gain provided an improvement, but also resulted in a reduction of contrast from the videcon tube. Disarming the automatic gain did not completely eliminate the gain shifts since illumination level changes still produced nonlinear responses from the videcon. A further complication was the light scatter in the lens that was built into the camera. The combination of the videcon and lens produced a nonuniform image that varied smoothly from the maximum brightness at the center to 50% of the maximum at the edge of the image. To eliminate the simple automatic gain circuits common in the lower cost cameras, solid state sensor devices were investigated. Since the DS65 was designed for NTSC video, the selection was restricted to standard video output devices as opposed to line scanning or optical RAM systems.

A series of simple tests was devised for selecting the solid state camera. First, the camera, without lens, was illuminated (using the lights and diffusing screens depicted in Fig. 2) and profiles were taken across the field-of-view to determine the uniformity of response of the basic sensor array. The lens and image sensors were then carefully cleaned and the lens installed. The camera was flooded with light and the analog image displayed on a monitor and inspected for any missing pixels or other blemishes within the sensor array. Our criteria was that the central 80% of the image had to be blemish free with no dropped pixels. A 2 × 2 pixel ROI was then selected near the center of the image. A black sheet of paper was used to block off the central 50% of the image and the black level was adjusted to yield a gray level of 2. The paper was then removed and the gray level set to 60. The average gray level in the ROI was then measured with 100% of the image field illuminated and with 50% of the field illuminated (leaving the ROI in the illuminated zone). Our criteria was that the gray level shift had to be less than one part out of 60 (1.7%).

We finally selected two devices: a General Electric model TN2500 providing a 256 × 256 resolution sensor matrix and a Javelin model JE2062 providing a 320 × 320 resolution sensor matrix. Our laboratory currently uses the Javelin camera, while other systems within our institution use the GE camera. A Fujinon model C6 × 17.5 zoom lens (12.5–105 mm) with extension tubes was selected as a low-cost lens suitable for both cameras. During normal operation, the lens opening is kept fairly small (f8) in order to reduce any optical falloff at the edges of the lens.

SOFTWARE DEVELOPMENT

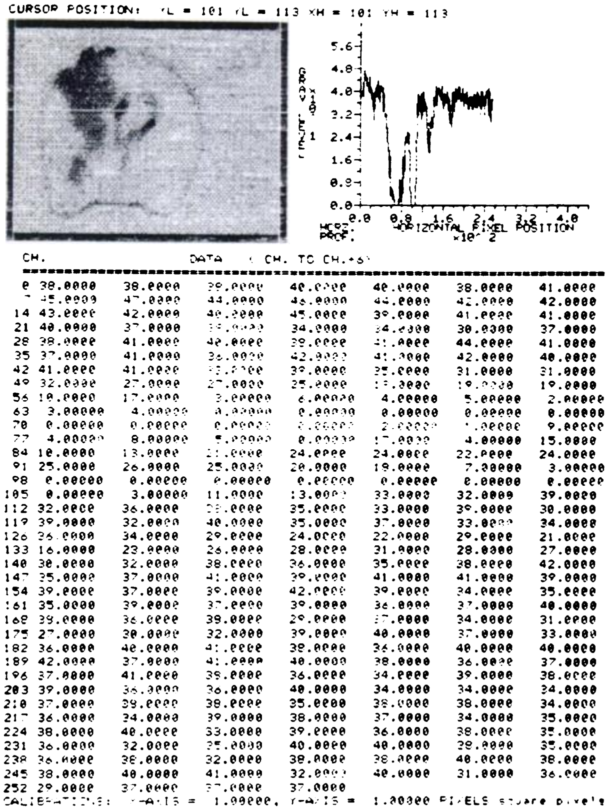
The software is written in Apple Pascal and 6502 assembler code. The current software functions supported are listed in Table 1. The principal machine-specific part of the software is the assembler code that controls the DS65 interface (the driver). This code consists of a single procedure which incorporates several different operations. The original version was based on a sample code provided with the DS65 interface which included reading pixel values and creating a ditherized display (writing patterns of dots on the Apple high-resolution display to give the illusion of a gray scale). The dither patterns used in the software are based on a square matrix with the pattern in the matrix selected by a lookup table. The user selects a 2 × 2, 4 × 4, or 8 × 8 dither matrix which results in a trade-off of the number of pseudo gray levels (4, 16, or 64) compared with spatial resolution on the Apple display or on the dot matrix printer output. The dither routine can be applied to data as it is captured by the digitizer or to an array of values (typically, data already stored on the disk). The dither package also includes a straight comparison where all pixels above a threshold are set to white and all pixels below the threshold are set to black.

TABLE 1
Video Digitizer Software Functions

1. Read individual pixel values (location selected by game paddles, user selected number of averages per reading).
2. Calibrate x-y pixels in units of distance (millimeters/pixel).
3. Measure distance between two cursors.
4. Digitize 192 × 256 image and create ditherized (pseudo gray scale) display.
5. Digitize 256 × 256 image and save it to disk.
6. Generate single profile (vertical or horizontal) across image.
7. Generate multi-line profile (vertical or horizontal) across image.
8. Generate rectangular ROI, then calculate mean, s.d., and range and display gray level histogram of data.
9. Acquire reference image for intensity renormalization (uniform response correction).
10. Renormalize digitized image.
11. Take calibration data to convert gray levels to specified units.
12. Print out ditherized image.
13. Print out profile or ROI data with ditherized image and plot of results.
14. Write profile or ROI data to disk file.
15. Select source for data (video without frame skip, video with frame skip, digitized image stored on disk).

When setting cursor positions for reading pixel values, measuring distances, or for setting profiles for ROI from realtime video, a white dot is superimposed on the video image which is viewed with a standard television monitor. The location of the dot is adjusted using the Apple II game paddle input. When using an image already digitized and stored on the disk, cursors are set using the Apple high-resolution graphics display and a ditherized image. Profiles and ROIs result in plots of the data and optional printer output (Fig. 3). In addition, numeric data files can be created to allow other programs to use the results conveniently.

In order to correct for nonuniform gain response from all areas of the video image, a renormalization system can be used. The routine is a straightforward multiplicative renormalization based on a reference image. The reference image is usually taken with a clean piece of film and the same illumination and settings of the camera f-stop used for the autoradiograph. In the case of a dropped pixel (a black pixel due to a defect in the camera) an average of the four surrounding pixels are used during the renormalization process. Thus,



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CURSOR POSITION:  XL = 101 YL = 113 XH = 101 YH = 113

  CH.                DATA   ( CH. TO CH.+6)
==========================================================================
   0 38.0000    38.0000    39.0000    40.0000    40.0000    38.0000    41.0000
   7 45.0000    47.0000    44.0000    46.0000    44.0000    42.0000    42.0000
  14 43.0000    42.0000    40.0000    45.0000    39.0000    41.0000    41.0000
  21 40.0000    37.0000    39.0000    34.0000    34.0000    30.0000    37.0000
  28 38.0000    41.0000    40.0000    39.0000    41.0000    44.0000    41.0000
  35 37.0000    41.0000    36.0000    42.0000    41.0000    42.0000    40.0000
  42 41.0000    41.0000    33.0000    39.0000    25.0000    31.0000    31.0000
  49 32.0000    27.0000    27.0000    25.0000    19.0000    19.0000    19.0000
  56 19.0000    17.0000     3.00000    6.00000    4.00000    5.00000    2.00000
  63  3.00000    4.00000    0.00000    0.00000    0.00000    0.00000    0.00000
  70  0.00000    0.00000    0.00000    2.00000    2.00000    1.00000    9.00000
  77  4.00000    8.00000    5.00000    0.00000   17.0000     4.00000   15.0000
  84 10.0000    13.0000    21.0000    24.0000    24.0000    22.0000    24.0000
  91 25.0000    26.0000    25.0000    20.0000    19.0000     7.00000    3.00000
  98  0.00000    0.00000    0.00000    0.00000    0.00000    0.00000    0.00000
 105  0.00000    3.00000   11.0000    13.0000    33.0000    32.0000    39.0000
 112 32.0000    36.0000    38.0000    35.0000    33.0000    39.0000    30.0000
 119 39.0000    32.0000    40.0000    35.0000    37.0000    33.0000    34.0000
 126 36.0000    34.0000    29.0000    24.0000    22.0000    29.0000    21.0000
 133 16.0000    23.0000    26.0000    28.0000    31.0000    28.0000    27.0000
 140 30.0000    32.0000    38.0000    36.0000    35.0000    38.0000    42.0000
 147 35.0000    37.0000    41.0000    39.0000    41.0000    41.0000    39.0000
 154 39.0000    37.0000    39.0000    42.0000    39.0000    34.0000    35.0000
 161 35.0000    39.0000    37.0000    39.0000    36.0000    37.0000    40.0000
 168 39.0000    36.0000    39.0000    29.0000    37.0000    34.0000    31.0000
 175 27.0000    30.0000    32.0000    39.0000    40.0000    37.0000    33.0000
 182 36.0000    40.0000    41.0000    38.0000    36.0000    40.0000    40.0000
 189 42.0000    37.0000    41.0000    40.0000    38.0000    36.0000    37.0000
 196 37.0000    41.0000    39.0000    36.0000    34.0000    39.0000    38.0000
 203 39.0000    36.0000    36.0000    40.0000    34.0000    34.0000    34.0000
 210 37.0000    39.0000    38.0000    35.0000    38.0000    38.0000    34.0000
 217 36.0000    34.0000    39.0000    38.0000    37.0000    34.0000    35.0000
 224 38.0000    40.0000    33.0000    39.0000    36.0000    38.0000    35.0000
 231 36.0000    32.0000    35.0000    40.0000    40.0000    29.0000    35.0000
 238 36.0000    38.0000    32.0000    38.0000    38.0000    40.0000    38.0000
 245 38.0000    40.0000    41.0000    32.0000    40.0000    31.0000    36.0000
 252 29.0000    37.0000    37.0000    37.0000
CALIBRATIONS:  X-AXIS =  1.00000,  Y-AXIS =  1.00000 PIXELS square pixels
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FIGURE 3
Profile of digitized autoradiograph of section of rat brain with tumor. Position of profile is indicated on ditherized image by white lines. Plot is data tabulated in printout. If gray scale had been calibrated, output values would be in μCi/cm^2

a missing pixel is replaced with an average value to be used in subsequent analysis.

A 64-element translation table to convert gray levels into other units based on calibration data is also available. The software allows the user to enter explicit conversion values for each gray level or let the system construct a table by digitizing an ROI in the autoradiograph of standards and entering the appropriate numerical value for the standard. The translation table assumes simple linear interpolation between entries on the table. The user can convert back to the standard gray level table at any time. When the conversion table is used, all outputs (numeric and graphic) are converted to the new units.

REPRODUCIBILITY

A major concern in any measurement system is the precision and accuracy of measurement. In a QAR system, there are many parameters involved. It is beyond the scope of this paper to discuss the problems in the selection of the radiolabeled compounds and the interpretation of the resulting distribution of that compound, or the optimal manner in which to integrate ROI or compare data from different sections of tissue. The major concern in terms of the video digitizing system is the ability to precisely measure optical density. To compensate for exposure times, difference in film response, differences in film exposure, changes in illumination of the film, and video gain in the camera, an autoradiograph of tissue sections must include radioactive standards (Fig. 4). Normally, these standards consist of methyl methacrylate plastic with known concentrations of isotope that bracket the expected isotopic density on the tissue sections. In this manner, the measured video gray level can be calibrated in terms of isotope concentration.

To test the ability of the microcomputer system to reproduce density values, the set of standards in Fig. 4 were redigitized over a period of 4 wk (20 measurements of each set). For each measurement series, the digitizing system was turned on, the film positioned, and the black level and contrast analog adjustments made by setting the darkest standard to level 3 and the lightest standard to level 60. The standard deviation of the measurement series (including seven standards) was 0.5 gray level. To indicate how well the gray scale calibrated with the standards related to tissue concentrations, a comparison of the methacrylate standards data to autoradiographs of brain paste was made. Figure 5 shows the calibration curve of the measurement of the gray levels compared with concentration from methacrylate standards exposed on the same film as the brain paste standards. While the response was nonlinear in terms of gray level (a function of both the normal log response

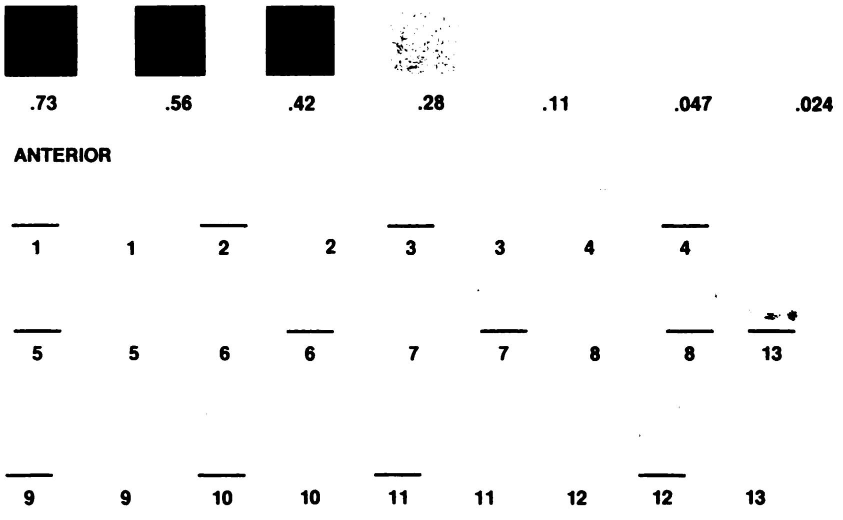

FIGURE 4
Sections of rat brain labeled with carbon-14 iodoantipyrine and killed 1 min after injection. Rectangular objects are methacralate standards to allow calibration of gray scale in terms of activity concentration

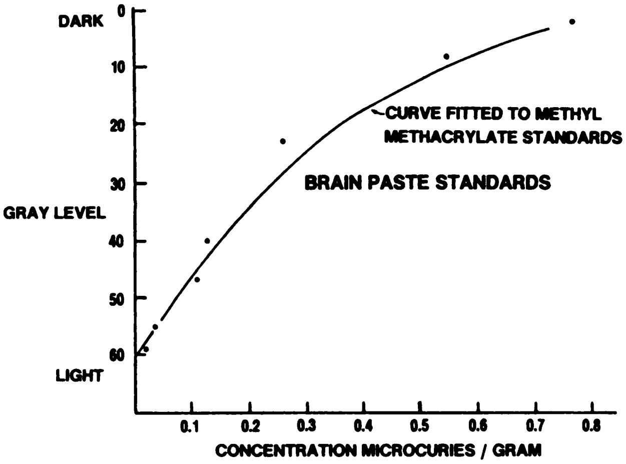

FIGURE 5
Comparison of curve derived from digitizing images of methacrylate standards (see Fig. 4) as compared with images of sections of labeled brain paste. Comparison was made to verify that methacrylate standards were equivalent to labeled brain tissue

of the film and the nonlinear response of the video camera), the calibration compared with the brain paste standards provides good agreement.

DISCUSSION

The system described in this paper has provided our laboratories with a low-cost, reliable method to perform quantitative autoradiography. The system has also been used for two-dimensional chromatography and cardiac ultrasound experiments. Other workers have found somewhat different solutions to the common problems of nonuniform light sources, nonuniform camera response, and suppression of noise. Some have found it necessary to use DC voltage light systems or high quality diffusing light sources such as slide copiers. While we have not seen a problem with our heavy filament lamps, any system must be carefully analyzed to determine if a nonconstant light output is producing artifacts in the images. The problem of nonlinear response and light scattering has resulted in the use of techniques such as masking the illuminated field-of-view to the size of the specimen and the use of more expensive cameras and high quality optical systems.

As discussed above, we did experience significant problems in terms of poor camera response and appropriate lighting. Since we have installed a lens hood in the camera to guard against stray light from sources outside the primary illumination, we have had very little problem with light scatter. There is the complication that the four lamps do not put out the same amount of illumination. The correction currently used is to place a shaped filter between the two diffusing plates. Usually the "filter" is a piece of black paper which is shaped empirically by looking at the digitized display. This approach has proven to be relatively easy to perform since the nonuniform illumination tends to vary slowly, presumably due to the use of two diffusing screens.

Since development of our system, many more microcomputers and video digitizing systems have become available commercially. Some commercial systems have recently appeared designed specifically for QAR and similar applications. Whatever system is chosen to implement QAR, care must be taken to assure that the practical problems are properly investigated. A major portion of such investigations must include the software—either the applicability of commercial packages or the effort required to develop such a package for a new system. In our case, the current software package represents over 2 yr of development.

Additional care in setting up such a system must be taken in allowing for expansion of capabilities. For example, renormalization requires a considerable amount of processing time, especially if the data files must be overlaid from the disk due to memory restrictions. Fortunately, several options exist for increasing the hardware flexibility of most microcomputers, including the Apple II. We are currently adding a 68000 processor[*] to our video digitizing system. This processor provides us with up to 2 megabytes of memory configured with ~80 kbytes for the system software and the rest as a virtual disk (RAM disk). Software is available for this system which emulates the Apple Pascal interpreter on the 68000 providing an increase in computation speed of 15–20. The system software uses the Apple itself as an input/output (IO) processor, and it is straightforward to add the video digitizer driver to the list of block IO devices. With this configuration, essentially all of our software will run without modification on the Apple/68000 combination.

Once the processing speed is sufficient to allow renormalizing a digitized image in 1 or 2 min, the major restriction in use of the system is the lack of a true gray scale display. Although there are now several microcomputers providing up to 16 colors per pixel, a true gray scale display with 64 or 256 levels of gray is needed to allow proper selections of irregular ROIs in digitized images. Many manufacturers are now making such display systems for selected microcomputers. We have experience with a set of displays[†] for multibus computers that we are adapting to the Apple/68000 combination for another project. This card set is particularly interesting since it includes a 512 × 512 × 8-bit video digitizer that digitizes an image in 1/60th of a second. However, for the existing video digitizing systems in use in our laboratories, we are adapting a 512 × 512 × 8-bit deep display made for the IBM personal computer by Number Nine Computer to the Apple/68000 combination. This particular display card uses a very simple interface and it is a straightforward matter to adapt it electrically to either the Apple or the Digital Acoustics 68000 bus.

It is clear from our experience and that of other investigators, that it is quite feasible to implement a quantitative autoradiography system on a wide variety of inexpensive microprocessors. The main problems with such applications are the time necessary to investigate sources of noise and nonuniform response in the camera/illumination system, and the effort required to develop an appropriate software package. With appropriate care and allowance for the software development time, it should be possible for most laboratories to develop similar systems. The software developed for the system described here will be placed in the public domain and made available through the Apple user's group of The Society of Nuclear Medicine.

FOOTNOTES

[*] Digital Acoustics, Santa Anna, CA.

[†] Matrox Corporation.

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