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Review

Colour thresholding in video imaging

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ABSTRACT

The basic aspects of video imaging are reviewed as they relate to measurements of histological and anatomical features, with particular emphasis on the advantages and disadvantages of colour and black-and-white imaging modes. In black-and-white imaging, calculations are based on the manipulation of picture elements (pixels) that contain 0–255 levels of information. Black is represented by the absence of light (0) and white by 255 grades of light. In colour imaging, the pixels contain variation of hues for the primary (red, green and blue) and secondary (magenta, yellow, cyan, pink) colours. Manipulation of pixels with colour information is more computer intense than that for black-and-white pixels, because there are over 16 million possible combinations of colour in a system with a 24-bit resolution. The narrow 128 possible grades of separation in black and white often makes distinction between pixels with overlapping intensities difficult. Such difficulty is greatly reduced by colour thresholding of systems that base the representation of colour on a combination of hue-saturation-intensity (HSI) format.

Key words: Colour imaging; colour thresholding; video microscopy; objective quantification; fluorescent in situ hybridisation.

GENERAL CONCEPTS OF VIDEO IMAGING

A short review cannot possibly cover all the basic concepts of video imaging microscopy, particularly the characteristics of different imaging techniques that are well exemplified in the publications by Mize et al. (1988), Kamiya (1991), Fermin et al. (1992) and Dewald et al. (1993). The following books are strongly recommended for anyone starting to work in this field: Inoue (1987), Toga (1990) and Shotton (1992).

INTRODUCTION

Applying computer technology to the analysis of biological specimens was made possible by the advent of fast and inexpensive microprocessors. Computerisation of morphometry reduced considerably the time previously spent doing manual measurements. Lack of automation and speed are the main problems of classic morphometric techniques (Weibel, 1969). Morphometric analyses (Bookstein, 1990) that required many years to complete in the recent past (Fermin & Igarashi, 1987) are now easily accomplished in just a few weeks.

With the flourishing computerised video technology also came the confusion of new and often non-standardised terminology. Reference to any book on video imaging will show a long list of acronyms, terms and definitions that are incomprehensible to the noncomputer specialist, and to those who are not experts in microscopic techniques. Even worse is the lack of standardisation of the mathematical algorithms and terminology used, a problem best illustrated by the various image formats now available (e.g. TIFF, PICT, JPEG). The first task facing those wishing to apply video imaging to their research is to master the terminology either by reading a suitable reference book (Inoue, 1987; Toga, 1990; Shotton, 1992), or attending one of the many video imaging workshops and courses offered.

Once the terminology is understood and a good grasp of computer usage is obtained, it becomes clear that image analysis is rather simple. What is complicated are the mathematical functions needed to extract

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information from an image. Fortunately many of these functions have already been developed and are available in public domain share-ware and from commercial companies. The way in which filters manipulate adjacent pixels has recently been reviewed (Hilman et al. 1990). Basically any image displayed on a monitor is made up of thousands of picture elements (pixels). The more pixels per unit area the higher the resolution, but with the increase in resolution, there is a trade-off in terms of the time needed to process the information. Manipulation of pixels in the image constitutes image processing and it will not add but only enhance, information (Linder, 1990).

ESSENTIAL HARDWARE AND SOFTWARE

Besides the standard microscopic equipment of the anatomist, a good computer, monitors, video boards and cameras are needed for imaging. If large areas (e.g. gross anatomical dissections) are needed the camera is attached to a standard or macrophotographic lens. Certain applications and software packages require a tablet for tracing objects. However, most video systems now available permit direct tracing of areas directly on the image screen with the aid of a mouse. Regardless of the cost of the system, the image is acquired with an analog or a digital camera, and the acquisition is made possible by an image storage video board. The image is then sent to monitors for display. Monochrome 8 bits systems usually first immobilise the image before the pixels within are manipulated. Colour systems such as V150 permit manipulation of the pixels in real time. Thus the options chosen for a particular video imaging system depend on the budget available. Most monochrome systems depend on video boards with 8 bits of information per pixel depth. High end systems can squeeze 24 bits of information in each pixel. Systems able to discern large number of differences between adjacent pixels therefore have higher colour resolution, require unique and occasionally dedicated computers and usually cost considerably more than systems able to squeeze 4–12 bits of information per pixel.

COLOUR AND BLACK AND WHITE THRESHOLDING

Thresholding refers to the separation of pixels into groups with similar characteristics. In monochrome systems such separation is dictated by the bits of information contained within each pixel. In an 8 bit system there are 256 possible permutations (n^8). The total value of possible grey tones can be divided into segments divisible by 8 in 'look up tables' (Nawfel et al. 1992). The more divisions possible within a given table the greater the number of differences that can be discerned between adjacent pixels. With 256 levels, there are 32 grades of separation. The lesser number of differences that can be separated between adjacent pixels is the factor that restricts 8 bits monochrome systems in separating unrelated hues of similar intensities. For instance, separation of the dark brown DAB chromogen of immunohistochemical reactions from a dark blue haematoxylin counterstain of a histological stain is possible but laborious.

Colour systems such as Oncor's V150 used to generate the photographs of this review usually contain more than 24 bits of information in each pixel. Thus a 24 bits system (n^{24}) is theoretically capable of generating tables with millions of possible colours. Separation of hues, and thus of differences between adjacent pixels, becomes easier but only if the computer housing the system is capable of performing the calculations needed in a reasonable length of time. The increased amount of information per pixel usually generates images that require more than 1 megabyte of storage space. Manipulating pixels of a colour image that contains more than 16 bits of information in each pixel at a quasi real time rate is very computer intense. Generally, special video boards are used to keep up with the demands of the calculations. The V150 referred to here uses parallel video boards installed on any personal MS-DOS based machine for making possible the calculations which deliver colour thresholding in a real time basis (Castleman, 1992).

IMAGE QUALITY

The quality of an image produced by any video imaging system depends on (1) the acquisition device (e.g. a camera), (2) the video boards that process the image, and (3) the output device that displays the image (e.g. monitor, printer). Thus once prospective users are committed to a particular level of resolution, all components must be of comparable resolution for satisfactory results.

Resolution

Colour systems are capable of spatial and colour resolution. Spatial resolution determines the ability of a system to resolve clearly 2 adjacent objects. Colour resolution is the ability of a system to separate closely related hues from closely related primary and/or secondary colours. The quality of an image is determined by the number of pixels in the image.

Pixels are like bricks on a building wall. They are picture units that result from the intercepts of the horizontal and vertical lines that make up each image. The more pixels per square area available the crisper the image appears and the better the resolution. A system with high spatial resolution may not resolve different colours without filtration. Some monochrome systems have more than 1000×1000 pixels resolution. However, these devices have standard video formats and may not be capable of processing in real time.

The amount of information contained in colour images usually restricts the resolution that can be produced. Monochrome systems do not have to deal with hue saturation, but rather with intensity alone (Fermin et al. 1992). Colour systems, on the other hand, must keep track of the hue and saturation information that is contained in each pixel in order to generate true colours.

There are colour charged coupled devices (CCD) capable of producing 750 TV horizontal lines resolution. These devices are expensive and do not have sufficient sensitivity (as measured in lux) to detect faint signals of true colours in real time. They can, however, integrate the image and produce a true colour image from signals that are sometimes too faint even for the human eye to detect. The V150 uses this approach for the analysis of faint fluorescent signals such as those obtained by labelling a single gene on a single chromosome by the so called FISH or fluorescent in situ hybridisation (Dewald et al. 1993; Kuo et al. 1994).

An analog video signal such as those of commercial TV sets can be displayed directly on the monitor or TV screen. This TV format is termed National Television Standard Committee (NTSC). The NTSC American standard format will yield a maximum resolution of 768×521 pixels. European PAL system yields a better 780×550 pixels resolution than the NTSC format because PAL registers the image in adjacent lines rather than in interlaced lines. The obvious choices for high resolution colour imaging are the RGB and HSI formats. Generating colour images with RGB requires extensive computer manipulation of the 3 separate inputs (1 for red, 1 for green and 1 for blue), whereas generation of colour images by HSI is less computer intense. In addition, in the RGB mode saturation of colours is detected in each channel separately. Yellow, for instance does not exist in the RGB model and when representation of such a colour is needed, it must be extracted from the primary colours by computer algorithms (Hills & Scott, 1990). Excellent reviews of primary and secondary colours have recently been published (Wells et al. 1992, 1993) and are recommended. Another excellent introduction to the properties of colours as they relate to photomicrography is found in Time-Life (1970).

The quality of any colour image depends greatly on the manner in which the image is generated. Colour alone is of little use without proper resolution (for a review of what resolution means for imaging, see Goldstein 1992). A good colour image is of little use if the pixel to pixel distance does not permit resolving the 0.2 μm distance dictated by photons in light microscopy. The importance of resolving power of the microscope combined with the resolving power of the video imaging device was elegantly discussed recently and these reviews are recommended as an introductory reading by Hilman et al. (1990), Castleman (1992). Assume that the purpose of an experiment is to measure the displacement of a gold particle on a moving muscle viewed on a TV screen with $\times 1000$ final magnification. At this magnification, a line on the screen of 10 mm corresponds to 10 μm. The pixel to pixel distance on the screen must be able to resolve the displacement or the data will be of no significance.

Intensity

An ideal CCD colour camera should have very high detection capabilities, and ideal biological preparations should have high signal to noise ratios; usually the opposite is true. As was mentioned above, high resolution colour CCD cameras are not yet able to image faint colour signals in real time, and this is one of the penalties paid by high resolution colour systems. The shortcoming is made up, however, by adding to the video boards the capability of image integration. The V150 microprocessors allow 1–999 integrations, making it possible to capture colour signals so dim that they are imperceptible to the human eye. This is the capability that was until recently restricted to intensified SITCON and other tube analog cameras (Inoue, 1987).

Special computer program subroutines (macros) are also added to colour systems to permit their automation, with the production of image frames that can be recorded, for instance, in standard VHS formats. We were successful in transferring faint fluorescent images to VHS tapes from where rationing of ions was possible without loss of information. Alignment of different images pixel by pixel was made possible by a subroutine for superimposing the current frame buffer over the saved frame buffer. Acquisition of dim or faint colour images in real time with colour

CCD cameras will remain challenging so long as high resolution and low detection capacity go in opposite directions. At present the higher the resolution, the lower the capturing capacity for displaying live colour images of faint signals.

Signal to noise ratios

These are expressed as the ratio between 2 signals, and demonstrate the significance of a signal of interest when subtracted from the background noise. The level of detectable noise of a system depends partially on the nature of the signals used. In particular, the manner in which signals from different electronic sources are synchronised is a very important part of reducing video noise. Loose connections, a broken wire inside cables, contaminated contacts, lack of grounding and incompatible signal formats are the main sources of noise and lack of synchronisation between signals. An ideal imaging device is one with high signal to noise ratio. A CCD colour camera with at least 1000 horizontal lines and capable of detecting signals of less than 0.1 lux intensity in real time would be a good candidate. Such a device would permit noise-free imaging of the faint signals such as those generated by Normarski interference contrast, fluorescence microscopy, etc. At present even phase contrast microscopy does not produce strong enough signals for live display with CCD colour cameras. When faint signals such as those above are viewed with colour cameras, a net increase of noise is seen on the TV screen as intermittent speckles also known as 'image snow' (for more information on this topic, see Inoue, 1987).

Input-output devices

Devices sending signals to the video boards of the video imaging system are designated as input devices. The common input devices are CCD cameras, video tube cameras, intensifier tubes and solid-state sensors. However, images digitised in other systems and saved in commonly used formats such as TIFF and JPEG, and read directly from storage devices, magnetic tapes and optical disks, can be manipulated on most video imaging systems. Digitised images usually retain their original resolution if the system reading the image is capable of similar or higher resolution. Analog signals such as those from VHS or PAL inherit the horizontal (lines/inch) resolution of parent devices, and such resolution is usually less than 300 TV lines/inch. The input imaging device determines the ultimate limit of sensitivity, wavelength dependence,and resolution of the entire video system, and for this reason it should be of the highest possible resolution and sensitivity. Cooled CCD colour cameras offer unsurpassed performance for low-level light conditions.

Devices able to display and record the images of the video imaging systems are designated as output devices. Some output devices accept signals directly from a computer, whereas others do so only after the digital signals are converted into analog signals (e.g. magnetic tapes). Here the danger exists that a high resolution image from a 24 bits colour system could yield a low resolution colour image from an intermediate lower resolution device. Thus, for an output device to reproduce the original resolution of a digital image, it must have similar or higher resolution. Colour printers such as the Sony UP5000 series produce high resolution publication-quality colour-corrected images similar to those reproduced here at the push of a button. The images can also be directly sent to an electronic matrix printer with 2000–4000 TV lines/inch resolution to produce extremely crisp projection transparencies from which prints can be made. If, however, the user needs a permanent colour negative record of the images, the same printers accept fine grain colour film such as Kodak or Fuji films. Newer negative film permits large amplification of images without considerable loss of details.

EXPERIMENTAL BIAS AND SYSTEM AMPLIFICATION

Before describing the most prominent features of the colour thresholding technique, we would like to note that no matter how expensive a system is, the basic principles of proper unbiased experimental design and selection of appropriate controls must be in place. Video imaging devices can produce huge amounts of numerical data, some of which may be of little use. Unfortunately the outputs from complicated manipulations are only as good as the input data.

Experimental design

The two major problems affecting quantitation with video imaging techniques are lack of standard recommendations for assigning numerical values to variables under analysis and lack of appropriate controls. This applies both to classic histological techniques and to more modern immunohistochemical and in situ hybridisation preparations.

In our laboratory we avoid bias by following a very simple routine during preparation of specimens that

are intended to compare variations of stain patterns. (1) All tissues are fixed, dehydrated and embedded under similar conditions (temperature, pH, osmolality, etc.) (2) The sections from different tissues are cut at exactly the same thickness and saved on manila folders, and sections from different cases are then placed on the same slide. (3) The slide with a built-in positive control is stained so that all the sections suspected of showing variations in staining patterns are processed at the same time and under identical conditions. When available the substrate for a particular antibody should be used to preabsorb the antibody to serve as a negative control. (4) Measurements are made from the sections on the same slide. In this way any small differences in measurement are probably due to real variations rather than to changes that are usually introduced by different preparative procedures or by small intra-individual variations. For instance, we found that identical dilutions of the same antibody can yield different staining patterns during different processing periods. Thus if the differences in which the main conclusion of a paper are based are very small, the manner in which the experiment was conducted comes into question regardless of the expense of the video imaging system used.

Extraction of data from digitised images can lead to improper and false conclusions if the variables under analysis are misunderstood. For instance, in a video system capable of measuring shape through thresholding of pixels within the area of interest, circularity (how round the cells are) may or may not convey information as to whether the surface of the cells is smooth or convoluted. Knowing how smooth the surface of a blood cell is can convey important information about its response to the osmolality of the solution in which the cells are immersed (Schön et al. 1991).

Limitations

It is very important to consider the limitations of the systems before starting a laborious experiment. First, it must be established that what it is wished to measure is within the limits of the system's resolution. Thus if the diameter of bacteria with an average 0.150 μm is to be measured, the system must deliver that resolution or better. Since the limit of resolution of the light microscope with oil immersion objectives and condensers of the highest possible numerical aperture (1.4) is 0.2 μm (this is determined by the wavelength of the photons of light) the first issue at hand to understand is that the remaining magnification needed will probably be an empty magnification enhanced by the digital devices. Secondly it must be possible to detect the differences visually that it is desired to measure, or the expensive camera may not see it either. Take, for example, tissue embedded in methacrylate and stained with a mixture of basic fuchsin and toluidine blue. The details of tissues and cells are useful for evaluating dimensional changes. However, the differentiation between cell types and between the nucleus and the cytoplasm by colours is almost impossible. Separation of these features with a video imaging system depends on the ability of the system to detect different intensities in monochrome mode, and different hues in colour modes. In either case, the separation of intensities or hues is possible but very laborious and is probably not worth the effort. Similar situations occur when attempting to count gold particles or silver grains when it is usually faster and cleaner to perform the counts by hand (Fermin et al. 1994). Thus advanced technology as described here has limitations that uninformed users may misunderstand.

Troubleshooting

The first thing that aspiring users of video imaging technology must be ready to accept is the ability to locate errors. This usually involves at least a basic knowledge of the computer system that houses the video boards. It is not unusual to spend many hours of discussion with the manufacturers or with colleagues trying to solve unexpected problems. This is particularly important when the software packages are pushed to their limits, or the system is employed in tasks other than those designed for use with macros and auxiliary modules. If the user is not willing to undertake these tasks, he or she can expect to pay dearly for others to solve the problems. Last but not least, video imaging is in its infancy and changing at very rapid pace. Thus frequent upgrading of hardware and software can prove to be highly expensive. For instance, upgrading of the video board may require a faster computer and higher resolution monitors, whereas staying with the older software may leave the user without technical support from the manufacturers.

TRUE COLOUR/REAL TIME IMAGE ACQUISITION

The discussion has so far dealt with the general properties of video imaging regardless as to whether the system is monochrome or colour. When an image is frozen or captured by means of the video board, it

is generally translated into a digital format and for all practical purposes becomes a binary array. Special headers at the beginning identify the image as TIFF, JPEG, GIF, etc., just some of the more than 50 different formats available today.

Once in these formats, the images are easily manipulated by most imaging systems at present available, through the use of standardised mathematical algorithms known as filters. As discussed above under resolution, pixels are picture elements (units) that contain the information of the image. Adjacent pixels can be compared and enhanced by the filters. Most inexpensive video imaging systems, including several share ware programs such as NIH-image, take advantage of these filters to manipulate the image. The original signal of the image is usually not deleted, but instead is enhanced (e.g. more black and less white) or cleaned (e.g. to remove dirt particles).

At this point it is important to distinguish between image processing and image analysis (Schön et al. 1991). Image processing improves the image with filters which may include contrast enhancement, pseudocolour, edge enhancement, etc. These filters usually produce the same amount of information in the output as was contained in the input. In image analysis the output is not always the same as the input. Area, perimeter and the shape factor of cells from a given image are numerical representations of the image analysis performed.

Generally only advanced computers and imaging stations are able to manipulate pixels with colour information in real time, because most colour images occupy more than 1 megabyte (MB) of space. Monochrome systems with up to 12 bit resolution have only to keep track of 12 pieces of information in each pixel. However, in a 24 bits system capable of delivering high resolution HSI images, the computer must keep track of 24 levels of information for each pixel. On an image with a resolution of 700×512 pixels, the computer must be fast enough to process each bit of information within a reasonable length of time.

ANALOG AND DIGITAL SIGNALS

We are surrounded by an analog world in which the information is represented in the form of continuous amplitudes. Computers, on the other hand, see things in digital form as arrays of zeros (off) and ones (on) signals. A binary system such as that used in the biological world by a planarian worm, for example, sees everything as either black or white, in much the same way that a photocopying machine copies text characters onto paper. Manipulation of pixels containing such limited information in each pixel is straightforward, but the same is not true for high resolution imaging systems.

The images from a gross anatomical sample, via a microscope or from a photograph, are perceived as analog signals. Once reaching the computer video board and translated to pixels, the image becomes digital and is then amenable to image processing. Nevertheless, the imaging system used must be able to read the format in which the information is stored. Some video systems have built-in computer programs that allow interconversion of image formats, while others make this an expensive option. It is important, therefore, to question whether the system under consideration permits saving images in a storage device as in one of the common formats in use today (TIFF, JPEG, GIF, PIC, etc.).

The video imaging system used to generate the images shown in this review produces RAW, TIFF and default image formats. The images are of the HSI type with more than 450 TV lines of resolution and were printed on a Sony UP5000 video colour printer. These images are an analog representation of digital files, are saved with a depth of 24 bits per pixel plus 8 bits graphic overlay, and each uses 0.7–0.95 MB of disk storage space.

Colour hues

An ideal video imaging system sees and manipulates images in much the same way the human eye and brain perceive colours. The V150 system that was used to generate the images shown in this review operates in a similar way to the human eye. The primary colours red (R), green (G) and blue (B) = RGB are seen by the rods and cones of the human eye, and interpreted by the brain as hue (H), saturation (S) and intensity (I) = HSI. Excellent descriptions of primary and secondary colours can be found in recent reviews (Wells et al. 1992, 1993). More in depth information about the characteristics of colour are to be found in Time-Life (1970). In anatomy, a distinction between closely related hues (pink, red, yellow, magenta, etc.) is important, as many stains produce these and other hues.

Hues (H) refers to the spectral colour or dominant wavelength (e.g. red, yellow, green, blue, cyan, magenta, etc.) Saturation (S) represents the degree to which white is mixed with the dominant wavelength. A highly saturated colour is spectrally pure, while a desaturated colour is mixed with white. A good

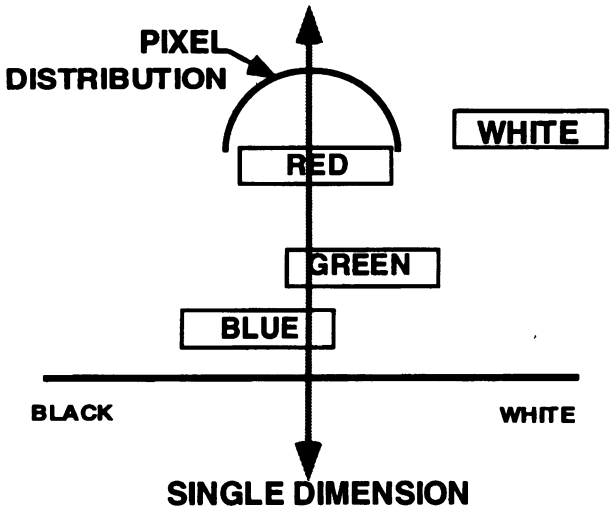


Fig. 1. In the monochrome mode of imaging there is an overlap of unrelated hues with similar intensities, so that red and blue, for example, are detected in a single channel.

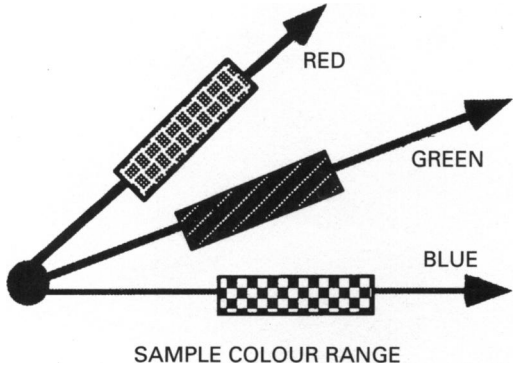


Fig. 2. In the RGB mode, primary colours are detected in different channels, but secondary colours do not exist and instead are deducted by a mathematical algorithm from the 3 primary colours.

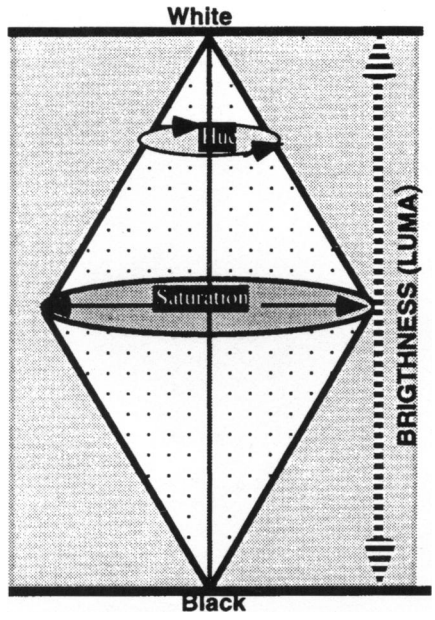


Fig. 3. In the HSI mode, a colour cube is formed in which the primary and secondary colours are represented because the relationship between saturation, hue and intensity is adjusted constantly (modified from Fermin et al. 1992).

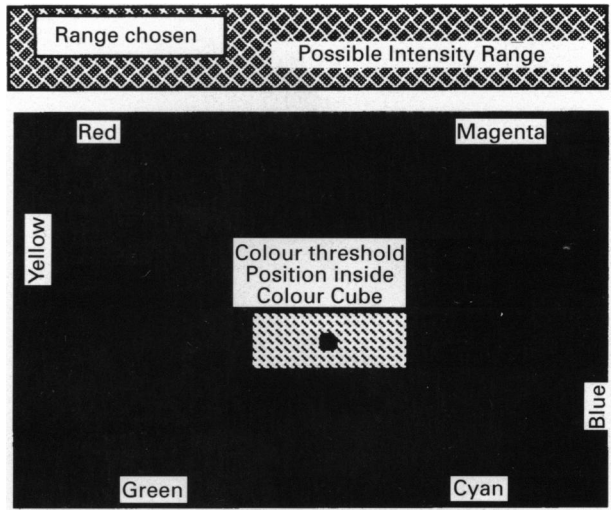
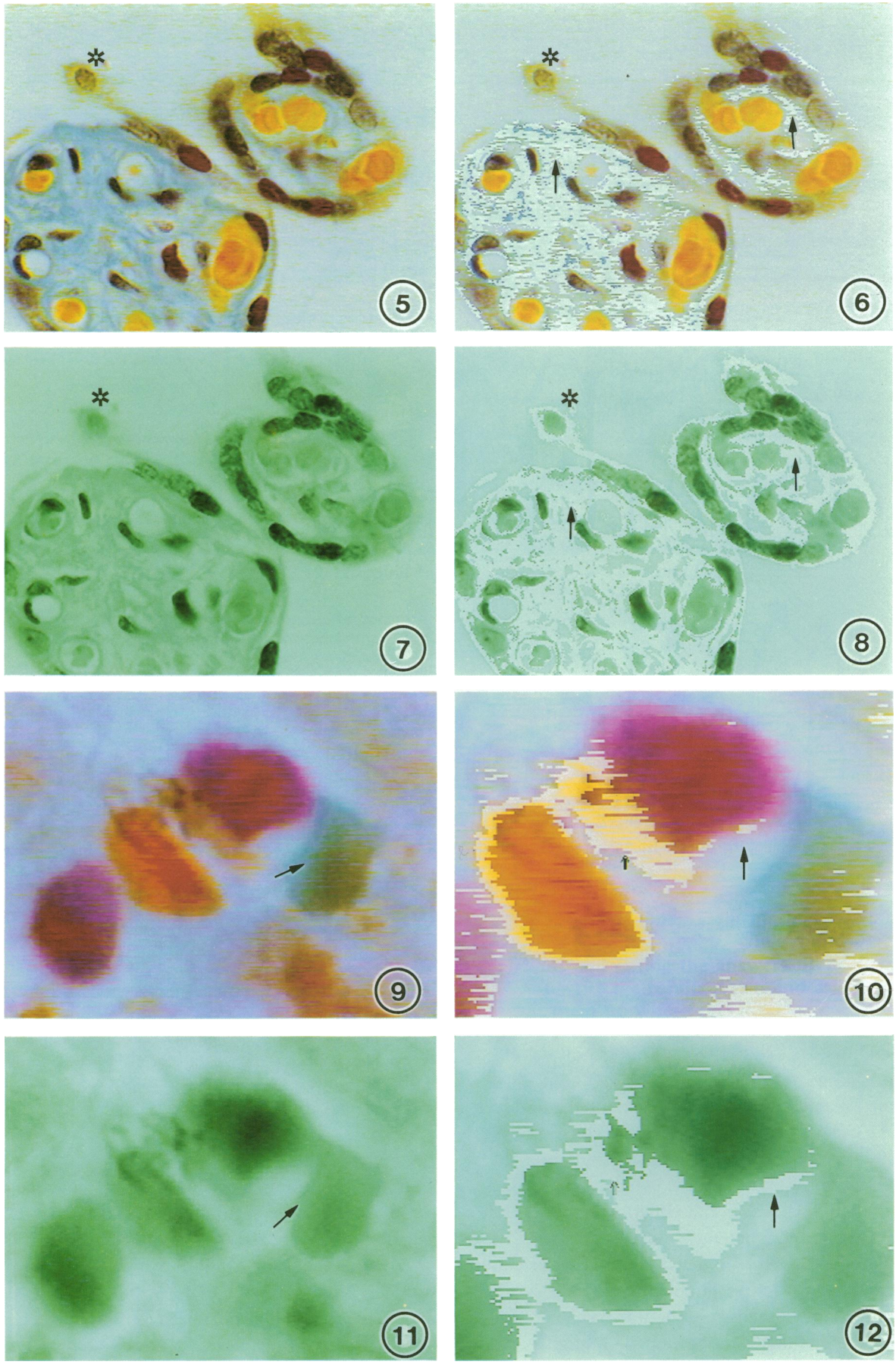


Fig. 4. A 2-D representation of the 3-D cube shows that primary and secondary colours that are available to the user either via the keyboard or preset functions of the system. Such flexibility permits the separation of very subtle differences between adjacent pixels (modified from Fermin et al. 1992).

example is pink which represents a desaturated red. Intensity (I) represents the intrinsic reflectivity of the object viewed, or the number of photons that are received by the camera. Figure 1 shows that monochrome systems cannot separate the characteristics of true colours even with filters, as filters only select hue and not saturation. This shortcoming of monochrome systems makes it difficult to separate fluorescent signals of closely related wavelengths. Thus, regardless of the spectra of two different fluorochromes, a monochrome system will show two bright white spots.

It thus follows that in the HSI video mode, colours are not separated from each other as occurs in RGB video mode. The RGB signals received from a colour camera do not correspond directly to the hue, saturation and intensity (Fig. 2). The unwanted consequence of this is that a change in brightness will change all 3 colour channels without discrimination, and the saturation of each colour is encoded as a ratio. For example, saturated red is encoded as a high signal value in the red channel and zero signal in the green and blue channels. Pink would be a high signal value in the red channel and low but non-zero values in green and blue. The final representation of an RGB image on the output device is not as pure or as close to how the human eye sees the image as with the HSI



Figs 5–12. For legends see opposite.

format. Moreover, arithmetic manipulation of the 3 separate RGB channels for producing a colour image is very computer intense and therefore time consuming.

The way in which images are encoded by a video camera restricts the range of possible colours that can be represented. A colour video camera effectively forms 3 images: red, green and blue. All other colours are represented as combinations of these 3 signals. The method of encoding and displaying colours used in video cameras narrows the range of colours which can be displayed. A video monitor cannot display a saturated colour with as much brightness as it can display bright white because a white pixel is the result of maximum energy in all 3 colour channels while a fully saturated red, for instance, is by definition energy in only one channel. In this way, the total number of photons emitted for a saturated colour will be less than that emitted for white. Conversely, black is represented by no signal in any channel. The net result is that it is difficult to represent a dark saturated colour on a video screen.

On the other hand, an HSI system like the Oncor V150 keeps track of objects of varying hues from multiple spectra (Figs 3, 4). With cooled CCD cameras it is now possible to discriminate subtle differences between stains of tissue samples, with a resolution similar or better than that of the figures shown in this report. Fluorescent in situ hybridisation (FISH) represents the best illustration of the ability of the system has to detect low level intensity signals. With this technique small fragments of DNA or cDNA are used similarly to immunohistochemical techniques to tag individual genes onto whole nuclei or individual chromosomes. It is possible to show multiple genetic loci on the same chromosome with different cDNA because the resulting image represents the actual hues that are seen by the human eye. This ability of the system makes it unnecessary for users to select filters a priori (which introduces bias) or add pseudocolour (which requires defining in order to consult tables used to generate the false colours). Finally, the reader does not have to be told the original colour of a black and white image, and the operation is easier and faster.

Flexibility and macros

In this part of the review we will discuss some of the capabilities of the V150 imaging system by discussing colour and black images generated after live and frozen thresholding of pixels. All images were obtained from human tissues stained with a polychrome mixture, and generously provided by Dr M. P. K. Shoobridge (Shoobridge, 1983). Selection of pixels shown in Figures 5–8 can be performed in real time, but manipulation of pixels shown in Figures 9–12, 19 and 20 is only possible on a binarised and immobilised image residing in the buffer of the system.

Different slides with identical hues from multiple experiments are easily identified by choosing the threshold of interest and then scanning the various slides in real time. The time saved could be substantial when large areas are to be scanned. Optional computer controlled microscope stages and specific short programs (macros) can automate the search and the analysis. For instance, counting cells which stain differently from thousands of other cells on a glass

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Fig. 5. Human placenta after polychrome stain with red blood cells in yellow and collagen in blue. Separation of closely related hues is unmistakable.

Fig. 6. This illustrates that thresholds for blue hues on this field turned most pixels on, which appear white (arrows). Note, however, that the darker blue above the arrow on the left was not chosen by the threshold setting used, nor was the light brown-yellow hue.

Fig. 7. Identical area in a monochrome mode of the V150 colour imaging system. Compare with Figure 5. It is clear that separation of closely related hues (light blue and light brown-yellow) is more difficult.

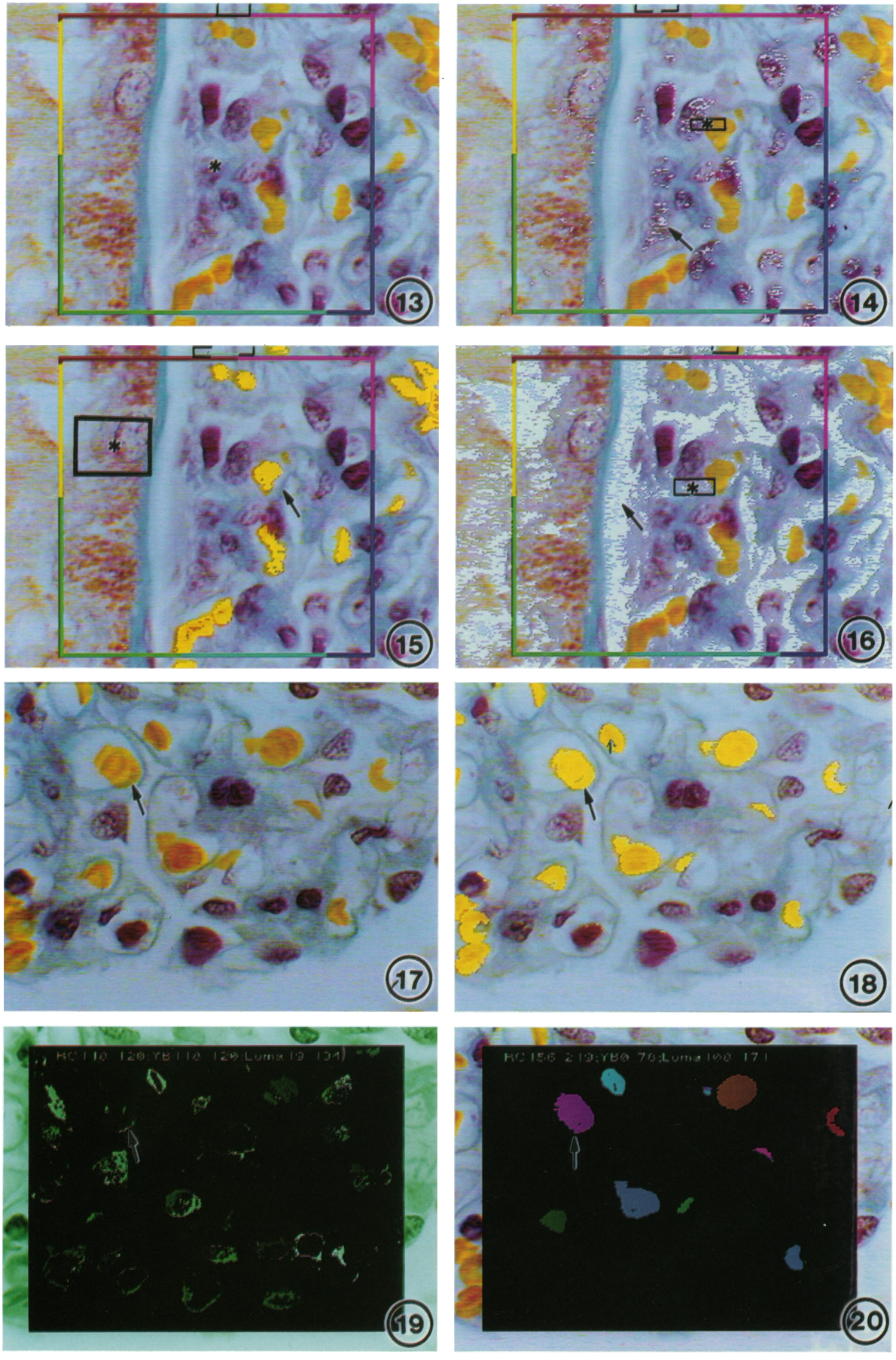
Fig. 8. Thresholding capable of choosing the pixels selected in Figure 5, with a similar colour thresholding scheme, also extends to closely related hues. The asterisk shows that the intensity of the cell cytoplasm is similar to the collagen in blue (see Fig. 1), and is thus not easily separated in a monochrome mode.

Fig. 9. Zoomed-in image of blood cells of human liver. A blue platelet is clearly separated from the overlying yellow hue (arrow) of this and other blood cells.

Fig. 10. Zoomed-in image showing horizontal line segments in white of pixels that correspond to the threshold setting that is necessary for choosing the yellow hue of these cells. Note that some pixels over the blue platelet are chosen, and similar colour thresholds around the red lymphocyte (arrow) were also chosen.

Fig. 11. This is similar to Figure 9, but in monochrome mode. Note that separation between the blue platelet and overlying yellow hue is more difficult (arrow).

Fig. 12. This is similar to Figure 10 and shows that similar thresholding as that used in colour failed to turn on exactly the same pixels as in the monochrome mode. In order to turn on the pixels of the main structure chosen in Figure 10, pixels of unrelated hues were also included (arrow).



Figs 13–20. For legends see opposite.

slide can be automatically accomplished if the user defines the thresholds and sets up a macro for that purpose.

Figure 5 is a HSI image of a human placenta without manipulation of the pixels in the image. Such an image is identical to images used during analysis. Most monochrome systems acquire images with a video board that can display colour images, but those images are generally converted to black and white before manipulations are allowed. Note that colours are crisp, the resolution of the image is quite good, the separation of tissue components is unmistakable and closely related hues (e.g. brown–yellow) are clearly separated. Figure 6 shows that the high resolution of the image combined with the crisp separation of related hues makes it possible to choose pixels that specifically represent a single colour, regardless of whether the saturation (intensity) of the colour is related to other colours in the same field. Figure 7 shows a monochrome representation of the same image. The first impression is that closely related hues were grouped even though digitisation was performed at 24 bits resolution. Note that the cell cytoplasm at the site of the asterisk is not different from the pale blue collagen in the rest of the image. In fact, thresholding in monochrome mode (Fig. 8) shows that in order to use the blue that was specifically chosen in figure 6, the brownish cytoplasm of the cell at the asterisk location was also chosen. Subtle differences such as this are encountered routinely in immunohistochemical preparations of poorly differentiated tumours, and are usually impossible to distinguish in a monochrome mode.

The reasons for the inability of a monochrome system to distinguish subtle differences are further illustrated in Figure 9 with its amplified (zoomed-in) image of blood cells in a small capillary of the liver. The pixels at the horizontal raster lines are now visible, and it is clear that hues are separated even when they are contained in overlapping pixels (see legends). Figure 10 shows that pixels that contain the information for a variation of yellow were chosen and are said to be in threshold, or turned on and are displayed white. Wherever pixels have identical thresholds, they are turned on without further manipulation, thus enabling the identification of the same hue in different slides. The thresholds are numerical values that can be entered into the system through the key board or chosen pixel by pixel with a mouse click. Figure 11 is a monochrome representation of the same image (compare Figs 9 and 11) showing that only part of the separation between the blue and the yellow hues is now possible. Because of overlapping hues, thresholding in monochrome mode (Fig. 12) to include features chosen before in Figure 10 will also extend to include objects of unrelated hues but of similar intensities, a problem that besets monochrome systems. Such a shortcoming is most pronounced when trying to separate areas of interest from background in dark field and immunohistochemical preparations.

Identical images of a glomerulus with a 2-D representation of the 3-D colour cube of the HSI system as a graphic overlay are shown in Figures 13–16. The box outside and above the cube represents the luma, which can change separately from the colours. In Figure 13, no thresholds are chosen (control knob at asterisk position) and no pixels are turned on. In Figure 14, the saturation of pixels

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Fig. 13. Human renal glomerulus with a polychrome stain. The 2-D representation of 3-D HSI colour space is superimposed onto the image. No pixels are turned white because the saturation and the luma (intensity) for the pixels making up this image are at zero (small asterisk at centre), i.e., there is an equal distance between the central axis of saturation and the representation of hues in the colour space.

Fig. 14. This shows that by increasing the saturation of pixels (small black box), and moving the threshold towards a red–magenta hue location, pixels representing the nuclear chromatin of some cells are chosen (arrow). However, colour objects of similar intensity are not chosen

Fig. 15. This illustrates that by further increasing the saturation with concomitant displacement of the box in the colour space towards the green–yellow hue, pixels for stained red blood cells (arrow) were specifically turned on even though other colour objects have similar intensity.

Fig. 16. Reducing the box to size near that of Figure 14, but not at the exact centre of the colour cube, turns blue pixels on without affecting other hues of similar intensities. In addition, a different saturation of blue (left of arrow), as for the basement membrane of the glomerulus, is not chosen with the threshold setting used in this case.

Fig. 17. This shows a renal glomerulus after polychrome staining with red blood cells in a yellow hue (arrow).

Fig. 18. Pixels for other cells with similar thresholds (Fig. 18) are also turned on (arrow), and even though the saturation of some pixels approaches the red end of the cube (below arrow), the threshold setting does not turn on pixels for similar related intensities.

Fig. 19. When the colour Figure 17 is binarised in the monochrome mode, detection of objects by colour threshold settings used to select features that are bright yellow in Figure 18 leads objects of unrelated hues but of closely related intensities to be chosen as well.

Fig. 20. In colour, similar thresholds yield specific identification of only those objects that fall within threshold chosen. Other objects with similar intensities but corresponding to different hues, and thus different thresholds, are ignored. Also note that objects within threshold, but at the border of the buffer zone for binarisation, are not included (lower left).

chosen was increased (black box with asterisk) and moved manually via the key board towards the magenta–blue axis. Such displacement of the control knob (central axis) enables (white pixels) the nuclear material of some cells to be chosen, while specifically ignoring other closely related hues of similar intensity (luma). When the central axis (knob) and the saturation it represents is displaced towards the opposite extreme of the colour cube (Fig. 15), the red blood cells stained yellow are chosen and pixels that correspond to that threshold are turned on. Interestingly, on returning the knob almost to a central position, but with different saturation (note box inside cube in Fig. 1) and altered luma (box outside and above cube in Fig. 15), some of the blue hues are also under threshold. The basement membrane is not chosen with this threshold setting even though it is also stained blue (Fig. 16).

Finally, Figures 17–20 show that colour thresholding can be used to select specific objects from within multiple colours, whereas a monochrome mode adds and/or subtracts objects of similar intensity but of unrelated hues. Figure 17 shows several red blood cells stained yellow inside a glomerulus. Pixels representing those cells are turned on selectively as shown in Figure 18. Instructing the system in monochrome mode to choose objects within the threshold set in Figure 18 (red–green 110–120; yellow–blue 118–120; luma 19–104) yielded multiple objects containing the thresholds set and the dimensions specified. Objects of unrelated hues but of similar intensities were chosen as well (Fig. 19). However, when the same analysis was done in the colour mode of the system, and with the above set threshold that specifically turns pixels for the red blood cells shown in yellow, only those objects within that threshold were chosen (Fig. 20).

These functions illustrated here can each be performed automatically by the execution of short program segments from within the Oncor V150 program. These macros provide a very powerful means of customising the system to perform unattended operations. The ease of development, execution and implementation of macros makes any video system flexible and ensures that it remains available in the market place for a long time since functions other than those already incorporated in the design can be added. We routinely execute macros that permit the determination of the ratio between different hues, and use them for comparing tumour versus nontumour areas of a biopsy specimen. In addition, we use macros that allow continued and repeated measures of pixels within threshold (e.g. the brown of peroxidase) inside a circle of known diameter. The system calculates the mean and standard deviation of the measurements, and results can be plotted for publication. Modification of macros provided with the system and a standard text editor, enables those with experience in the use of computers to customise the system rather easily. Macros intended for complicated and multiple tasks are also possible but may require assistance from the programmers at the company that designed the system at a modest cost.

CONCLUSIONS

The purpose of this review is to compare and contrast the properties of imaging system technology and specifically of monochrome video imaging systems with true colour, hue-saturation-intensity-(HSI)-based video imaging systems. Samples of screen displays are shown for each imaging mode to demonstrate that in histology there are subtle differences between cell or tissue types revealed by special stains. These differences are not always easy to separate with monochrome systems because they are often unable to separate hues of similar intensities (e.g. dark brown and dark blue). In addition, we discuss why red–green–blue (RGB) imaging based systems are more computationally intense than HSI-based imaging systems. The HSI mode is based on the same principle as used by the human brain for distinguishing colour. HSI consists of a 3-dimensional space within which discrimination of colours is performed without the need for filter selection. This is important because filters only select for hue but not for saturation, and the systems that rely on filters are slow and expensive. Differences between pixels that are impossible to separate with a monochrome system, such as those encountered in dark field imaging, are effortlessly separated with colour thresholding in real time. This is due to the fact that the HSI system can theoretically separate 16 million differences. Finally, the ability of HSI to preserve the hue and saturation of colours allows separation of fluorescent signals by the colour rather than by an a priori chosen set of filters. These properties enable the objective quantification of variables from special stains, immunohistochemical stains and fluorescence in situ hybridisation (FISH) stains, while preserving the actual hue of the stain used. The FISH technique is quickly gaining an important place in video imaging (Nederof et al. 1992*a*, *b*; Haar et al. 1994; Lanièce et al. 1994).

Video imaging is at present within the technical and the affordable reach of most researchers. Prospective

users must acquire basic knowledge of computer language and operating systems that run the computer where the system is to reside. Monochrome systems are not able to separate closely related hues of similar intensities without a priori (bias) setting of filters. Thus thresholding of colours of interest in histology may be difficult because of the overlap in intensity of unrelated hues, as for a dark blue and a dark brown. Monochrome systems are quite acceptable for basic morphometric operations and manipulation of pixels in a frozen image. However, if subtle differences between unrelated hues need evaluation, as is often required with special stains and immunohistochemical preparations, colour systems are a better alternative, because they permit separation of closely related hues. They are indispensable in the separation of very low intensity hues from low level signals such a those emitted by fluorochromes and in situ hybridisation, particularly if the two different signals are to be kept in their original form instead of separating them by the addition of arbitrarily assigned pseudocolours. Comparison of objects of similar thresholds but on different slides can be easily accomplished with colour thresholding in real time, and automated by application of specific macro programs.

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