

A Practical Technique of Colour Image Analysis: Applications in Experimental Research

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Abstract

Commercially available colour image analysers are relatively expensive. We describe a cheaper alternative developed by the Department of Experimental Surgery, Singapore General Hospital using an assembly of optical and computer equipment commonly available in the research laboratory.

This manual colour imaging system is comparable to the commercial model in terms of functional capabilities and accuracy, except that it takes a longer time to process and analyse images and is unable to measure colour density. However, it is capable of not only analysing microscopic images of stained histological tissue sections but also X-ray images and images of large pathological specimens. In the case of commercial models, different systems have to be used to analyse images from different types of specimens.

This system was developed in 1987 and has since been used successfully in a number of experimental studies. It has been applied to the measurement of parameters defining eye anatomical configuration, delineating the extent of tissue necrosis and fibrosis after therapeutic treatment and surgery, the development of new bone formation in fracture healing and to quantitative studies in liver regeneration. Due to its accuracy, low cost and versatility, this system should be within the means of even the most modest research laboratory.

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Introduction

Colour image analysers have been extensively used in medical research since 1970 for histomorphometric studies.¹⁻⁷ Image analysis gives more information and a better understanding of relative morphological changes in histological tissue sections. This is especially pertinent when different morphological areas are immunohistologically stained and present in contrasting colours or in the same colour with different intensities. In many types of experimental research, the use of some equipment for colour image analysis is in fact crucial to the study.

Commercial colour image analysers are unfortunately relatively expensive. The components of such an analyser can however be easily found among commonly available laboratory equipment. A collection of parts such as the single lens reflex (SLR) camera, photomicroscope, digitiser, and micro-computer (equipped with a software program such as the Autocad Release 12) can be easily assembled to function as a practical manual colour imaging system at minimal

extra cost to the laboratory. This manual colour imaging system would be capable of performing most of the parametric measurements a commercial model is designed to do. This includes measurements such as linear length, angular dimension, area, perimeter and three-dimensional reconstruction. Software programs such as the Autocad are however not capable of measuring grey scale and the colour density of images, a function which is available in most colour image analysis models available commercially. However, in some types of studies (e.g. the quantification of positive immunohistological stained cells), even the use of the Autocad Program is entirely optional.⁸

We describe here an example of an inexpensive and easily assembled version of a colour image analyser developed in our institution and in use since 1987. This particular version has been successfully used in a number of experimental research projects including the definition of eye anatomical configuration, delineating the extent of tissue necrosis and fibrosis after therapeutic

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treatment and surgery, the development of new bone formation in fracture healing and for quantitative studies in liver regeneration.

Material and Methods

The design of this manual colour imaging system (Fig. 1) is based on the principle of digitisation of projector images and subsequent analysis using the Autocad Release 12 program (Autodesk Inc, USA). The procedure involves 4 stages of image processing:

1. Image Capture

The specimen is tagged with a millimetre (mm) scale placed in the same horizontal plane as the specimen. This scale will be subsequently used for calibration during image analysis. Images of large pathological specimens and X-ray films can be directly captured by a single lens reflex (SLR) camera. A photomicroscope (Nikon FX-35DX camera system) is required for stained histological tissue sections or other microscopic objects. The film is then developed and the images are presented in the form of 35 mm photographic transparency colour slides.

2. Image Projection

A slide projector (Kodak Ektapro 5000) is then used to project the colour slides and the projected image is focused onto a digitiser tablet (SummaSketch II Professional Plus). The distance of the lens of the slide projector from the digitiser is fixed (after initial trial and error) to give a focused projected image which covers the whole digitising area of 330 x 420 mm.

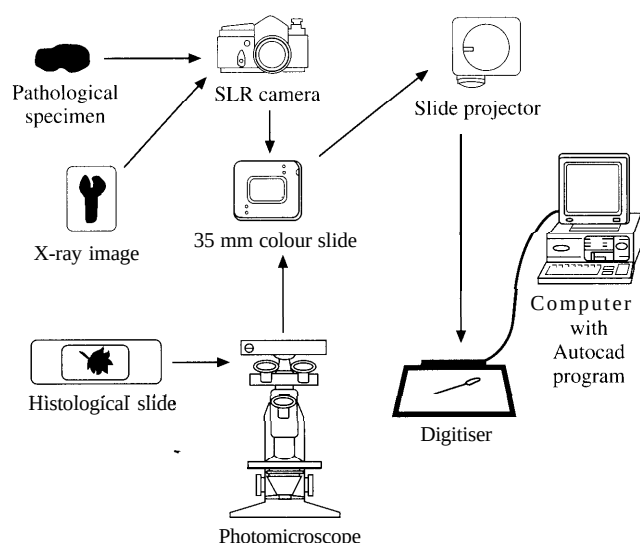


Fig. 1. Design of the manual colour imaging system.

3. Image Digitisation

The millimetre (mm) scale that was incorporated into the image is used for calibration of distance and all measurements in the projected image are expressed relative to this scale. The images projected onto the digitiser are generated on the screen of the microcomputer in a two-dimensional format and show up in different colours and shades. This allows areas of interest to be easily identified and mapped out with a mouse cursor.

4. Image Analysis

The images generated on the computer screen are viewed and distances measured using the AutoCad Release 12 program. This program allows for the measurements of length, area, perimeter and angle. The images can also be orientated at will, a function that allows for multiple images of a sequentially sectioned tissue specimen to be stacked up in layers and aligned along a reference plane to give a three-dimensional reconstruction of the specimen.

Alternative Arrangement

The mapping of defined morphological areas is not required in all studies that utilise colour image analysis. In immunohistological studies such as those involving malignant or regenerating tissues, it is frequently necessary to enumerate immunostained cells within a defined field and construct labelling indices.⁸ In this kind of studies, the use of the micro-computer and digitiser tablet is not required and images may be projected directly onto a plain paper screen.

Application

Histomorphometric studies: This manual colour imaging system is used to analyse stained histological tissue sections and specific morphological areas are mapped and measured. The system has been used to evaluate the extent of tissue necrosis or fibrosis after drug treatment or surgery, to evaluate new bone formation in bone remodelling during fracture healing and also to evaluate the degree of osteoporosis with measurements of trabecular bone area.

Anthromorphometric studies: In studies on the cadaveric femur, X-ray images of the femur are projected and neck shaft angles, femur lengths and angles and shaft diameters are measured. Similarly, photographic images of the hemisected eye can be analysed for corneal curvature and thickness, scleral thickness and anterior chamber depth after keratectomy.

This manual colour imaging system has also been used to produce three-dimensional reconstruction of tissue specimens where the entire architecture can be visualised to demonstrate branching and ramification of structures such as neurovascular bundles and ducts.

Liver regeneration: This system has been adapted to quantify rates of regeneration in the porcine liver after major hepatectomy.⁸ Bromo-deoxyuridine (BrdU), an analogue of thymidine is injected into the systemic circulation prior to hepatectomy. As the remnant liver regenerates, BrdU is incorporated into the nuclei of regenerating cells and can be demonstrated immunohistologically by a monoclonal-antibody system.⁹ Rates of regeneration at different time points and under different experimental conditions can be quantified and compared by enumerating the proportion of hepatocytes regenerating and constructing labelling indices.

In this application, the use of the micro-computer system is optional. Sectioned and immunostained histological specimens were first scanned by a Nikon (HFX-DX) light microscope under low power (with a 10x objective lens). A photomicrograph was then taken using a Nikon (FX-35DX) camera system, taking care to include at least one portal-triad area. This was then developed and processed commercially in the form of an Ektachrome transparency.

To identify and count cells, the Kodak Ektapro 5000 Slide Projector was mounted at a distance of 150 cm above an immobile stage and the image was projected onto a piece of white paper secured by tape. A portal triad was identified and a 90-degree quadrant of 15 cm radius centred at the portal tract and was marked out to include the immunoreactive periportal area (Fig. 2). Labelled and non-labelled cells can be easily identified and marked out by separate sets of symbols on the paper. The respective quantities of labelled and non-labelled hepatocytes were then ascertained. The labelling index, expressed as the percentage of labelled cells with respect to the total number of hepatocytes counted, was then computed as follows:

$$\text{Labelling Index} = \frac{\text{Number of labelled hepatocytes} \times 100}{\text{Total number of hepatocytes}}$$



Fig. 2. Photomicrograph of regenerating liver. Nuclei of regenerating hepatocytes are immunostained with BrdU and easily identified. Quadrant centering on a portal triad is superimposed on photomicrograph

The piece of paper onto which the photomicrograph was projected and on which the identification and counting of cells was carried out serves as a hard copy of the counting process and may be verified by an independent observer.

Advantages of the System

The most obvious advantage of this system is its minimal cost. This practical system also has a number of other advantages over the more expensive conventional models.

Commercial colour image analysers use software programs to identify colour images by differential colour wavelength and density selection. Differential areas are then marked out. However, in cases where colour contrast is poor or when colour densities tend towards the darker shades, the areas marked out can overlap and image edges are poorly demarcated. In such instances laborious editing procedures are required. Our colour imaging system allows input and judgement from the investigator. Differential areas are mapped manually and this allows less chance of erroneous image interpretation.

In the analysis of pathological specimens or X-ray film, the millimetre (mm) scale incorporated in the image is used for calibration and gives an accuracy correct to 1 mm linear length for lengths of even up to 100 mm and is more than adequate for image analysis. Comparison of values obtained using this system with that obtained separately with a commercial Leica Q500MC model shows comparable correlation and measurement values (unpublished observation).

Enumeration of labelled cells is traditionally done using a light microscope.⁹ This form of manual cell counting is known to suffer from imprecision, especially for the inexperienced observer. The liver in particular comprises more than just hepatocytes; endothelial cells, Kupffer cells, ductular epithelium and lymphocytes may confound accurate cell count. Indeed the majority of BrdU incorporation in the resting liver occurs in the non-hepatocyte cell population. The use of this projection system for the enumeration of labelled cells allows for a re-count of the exact same area by the same or a different observer. Furthermore, the higher magnification afforded by this method enables the easier distinction of hepatocytes from non-hepatocyte cells.

Discussion

This manual colour imaging system is an accurate measuring device and, although it is cheap, still gives measurements comparable to the more expensive commercial models. In terms of functional capability, the main disadvantage of this system is its inability to measure colour density. It also requires more manual manipulation and hence requires more time spent on the

part of the investigator. This very feature of the system, however, enables the input of good judgement and avoids erroneous interpretation of images. Another advantage of this system over commercial models is that a single unit can be used to analyse microscopic, macroscopic and X-ray images. Different commercial models would otherwise be required to analyse different types of specimens.

This manual colour imaging system is an assembly of commonly available equipment in the research laboratory and should be within the reach of all research laboratories. When not used for colour image analysis the different components can be conveniently disassembled and used for their originally intended purposes.

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