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Research paper

Validation of computer-assisted, pixel-based analysis of multiple-colour immunofluorescence histology

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Abstract

Developments in immunohistology allow the routine simultaneous use on tissue sections of three monoclonal antibodies, tagged with different fluorochromes. Such staining can identify seven different cell populations and the limiting factor is rapid, reliable and reproducible analysis. Future reliance on computer-assisted analysis of digitised images depends on validation against manual counting, often viewed as the 'gold standard'.

In this study images were digitised from sections of normal porcine skin, inflamed skin and tonsil, simultaneously stained with three monoclonal antibodies. Combinations of staining were quantified by four manual counts and by pixel-based area measurement. On individual images, the correlation between automated and manual measurements was poor. Despite this, the concordance between manual and automated measurements in the means and variances of tissues was good, and both techniques identified the same changes in inflamed versus normal tissues. In addition, pixel-based counting permitted statistical analysis of co-localisation of cell types in tissue sections.

We conclude that automated counting is acceptable for the assessment of tissues, is faster and provides less opportunity for observer variation than manual counting. We also demonstrate that the technique is applicable where more than three fluorochromes are used such that manual counting becomes essentially impossible.

Keywords: Immunofluorescence histology; Validation; Computer-assisted; Automation

1. Introduction

With the appearance of a wide range of antibodies to molecules expressed by cell subsets in all species, including the pig, it is now possible to carry out multiple-colour immunofluorescence and confocal histology. However, this powerful technique is limited by the need for quantitative analysis of images, particularly where more than three monoclonal antibodies are used concurrently.

The wavelength of sensitivity of the cones in the human eye, and the methods used for the creation of

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computer images dictate that the logical choice of three fluorochromes should be red (e.g. Texas Red), green (e.g. FITC) and blue (e.g. AMCA). In such three-colour (RGB) pictures it is possible to identify seven cell subsets: those staining red, green, blue, yellow (red plus green), magenta (red plus blue), cyan (green plus blue) or white (red plus green plus blue). The inclusion of a fourth colour, for example infra-red (IR), makes composite RGB-IR images essentially impossible for the human eye (or computer monitor) to visualise.

Manual cell counting can be sensitive and accurate in the hands of an experienced observer (Youssef et al., 1998). However, it is subject to inter- and intra-observer variation (Jagoe et al., 1991) and is time consuming (Bresnihan et al., 1998). Automated counting or assessment of the percentage area of stained tissue in a field has the advantages of reducing the observer variability (Kraan et al., 2000) and being more time efficient (Belien et al., 1999). However, it is difficult to design an algorithm for automated digital image analysis that is broadly applicable. Computer-assisted analysis also appears to be more susceptible to variations in staining than manual image analysis, presumably due to the ability of the human eye and brain to adapt and to recognise complex patterns (Johansson et al., 2001). Both manual and automated counts may potentially be affected by the background of the staining and other influences such as lamp and camera exposure settings (De Boer et al., 2001).

A colour digital picture is made up of a grid of picture elements (pixels) each of which have intensity values for red, green and blue. Many computer-assisted approaches involve initially identifying pixels whose colour intensities exceed a predefined 'threshold'. The approach taken after this point varies. One method for counting cell numbers is based on the identification of clusters of adjacent pixels with comparable colour intensities which can be used to define a discrete 'object' which can then be equated with a cell. This approach of object-based counting is the determining principle of analysis by laser-scanning cytometry (Oswald et al., 2004). However, it can suffer from a number of drawbacks when used for analysis of tissue sections: where cell density is high, individual objects may not be easily distinguishable and may not correspond to single cells; in tissue sections where cells are often partially stained, object-based counting may produce inconsistent results and limitations on the size or shape of acceptable 'objects' may need to be applied to avoid over-estimating cell numbers.

The alternative approach is simply to count the number of pixels whose intensity values exceed the appropriate threshold for each colour and to express this as a proportion of total pixels. The results of manual counting are usually presented as cells per unit area, and the results of manual, object-based and pixel-based counting will be directly equivalent if the area of a cell is a constant (or average) number of pixels (Reinhardt et al., 2001) and the area of a pixel is known (Eq. (1)). The potential disadvantage of pixel-based counting is that lack of co-localisation of staining within a single cell may result in pixels being assigned as single positive. However, these are comparable to the problems of object-based counting and the approach does not require the use of complex (and therefore fallible) algorithms for object recognition. We have previously shown that pixel-based counting of dual-colour fluorescence can provide comparable data to flow cytometry (Rees et al., 2003). Therefore, in this study, pixel-based counting was compared to manual counting using three-colour (RGB) immunofluorescence histology. In addition, pixel-based counting was used to assess statistical co-localisation of cell types within tissue sections and the technique was extended to demonstrate its applicability to four-colour immunofluorescence.

$$\frac{\text{No. of cells}}{\text{Total area}} \propto \frac{\text{Positive pixels/ pixels per cell}}{\text{Total pixels} \times \text{area of a pixel}}. \quad (1)$$

2. Materials and methods

2.1. Animals

The animals used for this study were healthy, 30-week-old Minnesota Minipigs killed with an overdose of sodium pentabarbitone, or Large White/Landrace hybrid animals killed similarly or by exsanguination after electrical stunning. All animals were maintained according to institutional guidelines.