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Appropriate Image Processing for Confocal Microscopy

Guy Cox[1] and Colin J.R. Sheppard[2]
[1]*Electron Microscope Unit, University of Sydney, NSW 2006, Australia*
[2]*School of Physics, University of Sydney, NSW 2006, Australia*

Most commercial confocal microscopes today are shipped with software capable of carrying out routine image processing. Typically, matrix convolutions are offered, working with either fixed or user-defined kernels. These allow crispening, smoothing and edge-enhancement operations, usually on square matrices of 3x3, 5x5 or 7x7 pixels. More sophisticated (and processor-intensive) operations such as median filtering may also be offered. All these operations are well known (Gonzales and Wintz 1987, Russ 1990) and are routinely employed where an image can be seen to benefit from the processing. However in current commercial systems they are applied only to two-dimensional images.

Confocal microscopes are generally used to acquire images in three dimensions, and in many cases (where physiological processes are followed through time) in four dimensions. One of the commonest uses of image processing on confocal images is to reduce noise, of which the main component in confocal fluorescence is photon sampling or shot noise. This, by its nature, will be randomly distributed in all 3, and indeed all 4, dimensions. Applying noise-reduction filtering only to individual two-dimensional slices will modify the noise distribution anisotropically, and is clearly not the appropriate procedure.

Effective noise removal requires filtering in as many dimensions as the data set contains (Chen et al. 1990). However, using a conventional square matrix this implies an exponential increase in the number of pixels to be processed at each point as the number of dimensions increases. A 3x3 matrix involves 9 pixels; 3x3x3 involves 27; if we progress to 3x3x3x3 then 81 voxels have to be used in computing each point of the output image. However square matrices are not the only posibility. Cruciform matrices, in which only those pixels adjoining the master pixel at a face are considered, are also often used in 2-D image processing. For 3-D and 4-D processing the cruciform matrix has the tremendous advantage that the increase is linear - each extra dimension adds just 2 more voxels to the number which must be handled at each point. We are therefore currently developing 3 and 4 D cruciform filters for confocal data sets, both matrix smoothing filters and a median filter. These offer the promise of a simple and rational noise removal technique for confocal data sets.

While shot noise is isotropic in all dimensions, other defects of the confocal image are not. The optical resolution, in particular, is isotropic in two lateral dimensions but substantially worse in the axial direction. The effects of this on a three-dimensional biological specimen are seen in Fig. 1 a & b. Because the point spread function of a confocal microscope approximates to an ellipsoid (Brakenhoff et al. 1989, Shaw and Rawlins 1991, Cox and Sheppard 1993), it is logical to apply the conventional tools of image processing in one dimension only - the long axis of the ellipsoid - to improve the perceived appearance of such images. Like all such operations, this will not increase the resolution or the information content of the image, but it will make it possible to extract information which would otherwise be obscured.

This approach was first applied by Cox and Sheppard, 1993. As a test case we used a simple edge-enhancing transform which detected the magnitude of the gradient at a pixel and used that as a weighting factor to modify the intensity of that pixel. It can be represented as:

$$1) I_n \leftarrow I_n - (|I_n - I_{n-1}| + |I_n - I_{n+1}|)/s$$

where I is the intensity of a pixel at plane n. The scaling factor s determines what fraction of the gradient is subtracted and must be matched to the degree of over or under sampling in depth. The effect on our biological image is seen in Fig. 1c.

A second transform is a simplification of (1), using only the values of the neighbouring pixels, in the interests of improving speed.

$$2) I_n \leftarrow I_n - (|I_{n-1} - I_{n+1}|)/s$$

This will have the same effect on the slope of the response curve but will behave differently at maxima and minima. In practice, its effect is generally visually indistinguishable from transform (1).

An alternative approach, again applying a classic technique of image processing, is to use an erosion rather than a gradient transform. A constrained erosion is used so that maxima and minima are preserved:

$$3)\ \text{IF } I_{n+1} < I_n < I_{n-1} \ \text{ THEN } I_n \leftarrow I_n - I_{n+1}$$
$$\text{IF } I_{n+1} > I_n > I_{n-1} \ \text{ THEN } I_n \leftarrow I_n - I_{n-1}$$

If a pixel has one neighbour higher than it and the other lower its value is replaced by that of the lower neighbour. The effect on an image is rather different from that of transforms (1) and (2); background haze is less well removed but the effect on the brighter part of the image is pronounced (Figure 1 d & e). Repeated iterations will 'skeletonise' the image, so that it could be used to find the true positions of multiple superimposed planes in the sample. This is not readily achievable in any other way.

All edge-enhancing transforms are susceptible to noise, so that ideally a noise reducing filter such as a median filter should first be applied to the data set. However applying this in the lateral plane only will be of little use - a 3-D filter is essential. Hence these two techniques, 3-D filtering and 1-D enhancing, are complementary. Providing implementations capable of adequate performance on the type of computers typically used to host confocal microscopes should not be difficult, and indeed some of these processes are already in common use by users of our facility.

The image processing operations presented here are conventional; what is novel is their application in a more rational way to the confocal image. For a 3D or 4D data set the combination of 3D and 1D processing is far more useful than the

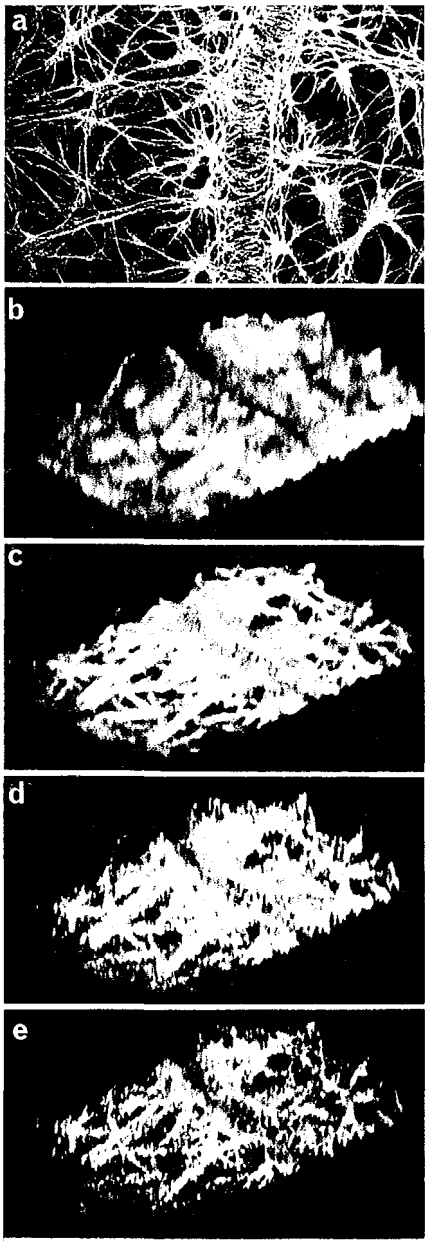

Fig. 1. 3D recontruction of rhodamine-labelled astrocytes around a capillary in the retina of a rat.
(a) Top view, showing good lateral resolution. The branches of the astrocytes are known to be isodiametric, and so should in a perfect image appear the same size in side and oblique views as in this top view.
(b) Oblique view of the original, unprocessed data set. The overall shape is visible but the image appears strongly smeared in the vertical direction; individual astrocyte branches can barely be distinguished.
(c) Processed with transform (1) - the appearance is dramatically improved. Most of the branches visible in (a) can be identified, though their depth still appears too large.
(d) Processed with the erosion transform (3) using one iteration.
(e) Processed with transform (3) using two iterations. The erosion gives more clarity in some areas, but a more 'ragged' overall effect, than transform (1).

2D-only processing offered by microscope manufacturers.

The techniques of image restoration, which also work in three dimensions, represent a different and not strictly comparable approach to the problem. An iterative approach is typically used with the aim of generating a model of the object which would, when operated on by the point spread function of the system, produce the observed image. These techniques were initially applied to widefield images, with the intention of creating optical section data sets without confocal optics (Agard et al. 1989, Carrington et al. 1990, Holmes et al. 1991). Latterly they have also been applied to confocal images, with the aim of creating a data set with enhanced resolution, particularly in the axial direction (Carrington et al. 1990 Shaw and Rawlins 1991, Wilson and Tan, 1993). Image re-storation is ambitious in scope, and demanding in computer power, but limited in its objective - the sole aim is to recreate the original object.

Image processing, by contrast, minimises unwanted information or enhances wanted information in the image. The unwanted information may equally be an artefact of the imaging process or a real part of the specimen; the wanted information may be all the 'real' objects in the image or a subset of them. Even if advances in computer power allow image restoration to become a routine part of confocal microscopy, there will still be uses for simple image processing methods - particularly if they are redeployed in a more appropriate form for the confocal image.

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