

Multimodal microscopy by digital image processing

C.A. Glasbey

Biomathematics and Statistics Scotland
JCMB, King's Buildings, Edinburgh EH9 3JZ, Scotland

and N.J. Martin

Biochemical Sciences Department,
Scottish Agricultural College
Auchincruive, Ayr KA6 5HW, Scotland

Abstract – A matching algorithm is proposed for aligning microscope images obtained using different modalities, making use of cross-correlations of outputs from Prewitt's edge filter. Brightfield, phase contrast and differential interference contrast microscope images of algal and bacterial cells from an experimental, high-rate algal pond are used for illustration. The information content of multimodal images is explored using principal components analysis and colour displays, and an image which represents optical thickness is constructed digitally.

Key words – Brightfield microscopy, Cross-correlation, Differential interference contrast microscopy, High-rate algal pond, Image matching, Optical thickness, Phase contrast microscopy, Prewitt's edge filter, Principal components analysis.

1 Introduction

Different imaging modes with the light microscope convey complementary information about a specimen. Brightfield microscopy primarily conveys information about the optical attenuation of the specimen, whereas phase contrast microscopy shows diffractive properties and differential interference contrast (DIC) microscopy responds to the refractive properties of the specimen. Bracegirdle (1993) provided a simple but lucid demonstration of this, revealing hidden details in old microscope slides by DIC microscopy. Similarly, Steinholt, Chandler and Tirado (1991) compared detection of the acrosome reaction of spermatozoa using DIC and brightfield microscopy, after staining. Chicino *et al* (1990) found that DIC microscopy had advantages over both phase contrast and brightfield methods for detecting *Pneumocystis*, although comparison of features of both the phase contrast and DIC images illustrated the potential advantages of multimodal microscopy. In an even more exhaustive comparison of microscopic imaging modes, Leonardi, Blakistone and Kyryk (1990) compared applications of polarised light, brightfield, DIC and scanning electron microscopy (SEM) in the paper industry.

Fluorescence microscopy adds further possible imaging modes to light microscopy. Germida (1984) concluded that non-specific fluorescent staining was better than brightfield and phase contrast microscopy for detecting spores of a microsporidian protozoan in soil. Collazo, Fraser and Mabee (1994) used images obtained with DIC and fluorescent vital staining to trace the embryonic origin of fish mechanoreceptors. In addition, many workers have exploited the ability of immunofluorescence to provide highly selective visualisation of microscopic structure. For example, Augustine, Watkins and Danforth (1992) used phase contrast, immunofluorescence and transmission electron microscopy (TEM) to investigate antigen release from antibiotic-damaged coccidian parasites.

At present the major application of multimodal microscopy involves fluorescence microscopy, either alone or in combination with brightfield, phase contrast, DIC or darkfield microscopy (Modrusan *et al*, 1994). For example, Ried *et al* (1992) proposed an elegant method which involved pseudocolouring and recombining images labelled with two visible and one infrared fluorochrome to apply seven gene probes to human chromosomes. Also, Armbrust, Ferris and Goodenough (1993) combined DAPI fluorochrome stained and immunofluorescent stained images to demonstrate the action of a chromosomal gene product on chloroplast nucleoid DNA in *Chlamydomonas*. A combination of darkfield and brightfield microscopy (at low magnification) was used for detecting gene probes in developing flowers (Jack, Fox and Meyerowitz, 1994). In discussing multimode microscopy, Farkas *et al* (1993) summarised the features of almost every conceivable mode of light microscopy. They

described integrated multimode workstations with advanced combinations of optics, several cameras and sophisticated software tools which control image acquisition, processing, analysis and display. However, they drew a distinction between such multimode systems and multimodality imaging, which involves combining images from spatially and/or temporaneously distinct sources.

The combined imaging systems considered above have the advantage that all imaging modes can be handled simultaneously, or sequentially, without changing major elements of the optical system. Additionally, the information conveyed by these techniques is sufficiently different that it is retained in the resultant optically-combined image. Optical image recombination is not an option for producing multimodal images which combine modes such as brightfield, phase contrast and DIC, because their optical requirements are mutually exclusive. Perhaps more importantly, the separate images must be produced and recombined in such a manner that the signal from one mode, for example brightfield, does not swamp the signal from other modes, where the overall intensity distribution may be very different.

In this paper we explore the application of digital image processing techniques as a potential solution to the problem of combining microscope images. Much effort has been expended in developing and modifying algorithms for matching images produced by different types of satellite remote sensing systems, such as optical sensors and synthetic aperture radar (see, for example, Richards, 1986, chapter 2). Recently there has also been considerable interest in combining images produced by different medical sensing systems in order to increase their usefulness (Colchester and Hawkes, 1991, §3). For example, X-ray images reveal structure, whereas magnetic resonance images reveal functionality. However, comparable developments specific to digital multimodal microscopy appear have been relatively neglected.

Methods are illustrated by reference to Fig 1, which shows images of a sample of algal and bacterial cells from an experimental, high-rate algal pond (Martin and Fallowfield, 1989), obtained using brightfield, phase contrast and DIC microscopy. Images were obtained on an Olympus Vanox microscope fitted with a Panasonic model WV-CL700 colour CCD camera. The brightfield and DIC images were produced using an Olympus Plan X40 0.65NA. flat field objective and the phase contrast image was obtained with an Olympus LWDCDPlan X40 0.55NA. long working distance objective. The photo tube was fitted with an Olympus FK X3.3 compensating eyepiece. The images were digitised on an Elonex 386 computer fitted with a VP1100-KIT-512-E-C1-AT Overlay Frame Grabber Kit image capture board using Optimas image processing software (both from Data Cell Ltd, 10 West End road, Mortimer Common, Reading, Berkshire RG7 3SY, UK). To avoid a strong interference caused by interaction between the frame grabber and the colour video camera, the frame size was set to 768 pixels \times 1024 pixels and subsequently cropped

to 512×768 pixels. Before storage, the images were processed, using the Optimas software's 'colour filter' facility to remove residual interference caused by the colour carrier signal from the CCD camera.

Before multimodal images can be combined they must be processed to compensate for changes in image alignment resulting from imperfect centration of the different lens systems, from slight movements of the specimen and from slight differences in magnification between objective lenses with the same nominal magnification. Galbraith and Farkas (1993) presented two methods for aligning images, the first of which required the imaging of a rectangular grid and the second the manual identification of control points. In §2 we present a fully automatic method of alignment. Then, in §3 the information content of multimodal images is explored using principal components, and an image, which represents the optical profile of an algal cell, is constructed digitally.

2 Alignment of images

Although the microscope specimen was not moved between modalities, the changes in optics of the microscope have produced some changes in position, particularly between the phase contrast image, Fig 1(b), obtained with the long working distance objective, and the other two images. The same objective was used in brightfield and DIC, but small image shifts were produced by inserting the Wollaston prisms into the optical train. It is important that these changes are corrected for, before proceeding to combine the images.

We represent a digitised image as a two-dimensional array of numbers corresponding to image intensities. We wish to find a transformation, which maps a position (i, j) in one image, where i denotes row number and j denotes column number, to (i', j') in another image. If the only difference between the images is a shift of location, either along rows, along columns or in some other direction, then

$$i' = i + i_0, \quad \text{and} \quad j' = j + j_0$$

performs the required mapping. Here, the top-left corner $(0, 0)$ of one image matches location (i_0, j_0) in the other image. If there is also some change in magnification between images, then

$$i' = ci + i_0, \quad \text{and} \quad j' = cj + j_0,$$

where the constant c performs a rescaling between images. A value of $c = 1$ corresponds to no change in magnification, whereas $c > 1$ is an enlargement and $c < 1$ is a shrinkage.

Yet more general transformations can be considered, to include rotations, differential stretching between rows and columns, and shearing. The most general linear transformation is the affine one:

$$i' = a_1i + a_2j + i_0, \quad \text{and} \quad j' = a_3i + a_4j + j_0.$$

Horgan, Creasey and Fenton (1992) used this mapping to superimpose SDS-PAGE gel electrophoretograms. In some applications in medical imaging and remote sensing, nonlinear transformations are needed, such as polynomials, splines and finite element methods. (For overviews of geometric transformations see Bookstein (1991) and Tang and Suen (1993).) With microscope images this would be required if significant distortion were present between images. The video images utilise only the central 50% of the visual field of the plan optics used here, however, so this correction is unnecessary and we will not pursue the matter further. A more serious problem arises if there is relative motion between particles within the picture frame; as for example will happen with motile microorganisms. This presents a much more difficult computational problem and for the purposes of this investigation only fields in which the cells were stationary were used.

Another issue to be addressed is how to choose the parameters in the transformation. One approach is to identify a few features which are common to the two images. Then a regression algorithm can be used to estimate the linear parameters which bring these features into alignment (Horgan, Creasey and Fenton, 1992). While satisfactory for images containing well defined punctiform details such as the fluorescent probes used by Ried *et al* (1992), this method is less than ideal for normal images. For the latter, it depends on subjective judgements and can be time consuming if it has to be done repeatedly. Therefore, a fully automatic algorithm is preferable.

A criterion of agreement between images is required, which an automatic algorithm can seek to optimize. The cross-covariance and cross-correlation are widely used measures. If $f_{i,j}$ denotes the image brightness at the pixel located at (i, j) in one image and $f'_{i',j'}$ denotes the image brightness at (i', j') in the second image, then the cross-covariance is proportional to

$$\sum_i \sum_j (f_{i,j} - \bar{f})(f'_{i',j'} - \bar{f}'),$$

where \bar{f} denotes the average pixel value in the first image and the summation is over the area of overlap between the images (see, for example, Rosenfeld and Kak, 1982, §9.4). Cross-correlations are obtained by dividing the cross-covariances by the standard deviations of the pixel values in the two images. The transformation $(i, j) \rightarrow (i', j')$ is chosen to maximize this criterion. If the transformation is simply one of translation, then the cross-covariance is a linear convolution, which can be

evaluated very efficiently for all possible changes in origin simultaneously using a Fast Fourier Transform (Glasbey and Horgan, 1995, §3.2). The array of cross-covariances is given by

$$\mathcal{F}^{-1}\{\mathcal{F}(f - \bar{f})\mathcal{F}^c(f' - \bar{f}')\},$$

where \mathcal{F} denotes the Fourier transform, \mathcal{F}^c denotes the complex conjugate of the transform and \mathcal{F}^{-1} is the inverse transform. If an unknown change in magnification is also involved in the transformation, then the covariances need to be evaluated for a range of values of c . Maximizing the cross-correlation was tried as an approach, but it did not perform well with microscope images obtained using different modalities. This result is not surprising, given the differences between images which can be seen. For example, in brightfield microscopy, algal cells appear dark, whereas in DIC one side of cells is dark and the other side is light.

Phase-correlation, defined as

$$\mathcal{F}^{-1}\left\{\frac{\mathcal{F}(f)\mathcal{F}^c(f')}{\|\mathcal{F}(f)\mathcal{F}^c(f')\|}\right\},$$

where $\| \ \|$ denotes the modulus of a complex number, was proposed as a matching criterion by Kuglin and Hines (1975). It performs better than the cross-correlations in applications where differences between images occur only at a subset of frequencies. This would be the case, for example, if the trend in illumination differs between the images, but not if significant levels of white noise are present. It performed better than cross-correlations with our microscope images, but not well enough.

We then considered using gradient images instead of intensity ones, because positions where intensities change rapidly at the edges of cells generally coincide in the different modalities. Edge information has been used by Bajcsy and Kovacic (1989) and Moshfeghi (1991) to align medical images. Prewitt's edge filter (see, for example, Glasbey and Horgan, 1995, chapter 3) provides a simple way to extract edge information from an image. More sophisticated edge filters, such as Canny's (1986), could have been used, but Prewitt's filter proved to be adequate for our purposes and is faster to compute. The edge strength at pixel (i, j) is calculated as:

$$g_{i,j} = \sqrt{y_{i,j}^2 + x_{i,j}^2}.$$

Here, $g_{i,j}$ estimates the rate-of-change in image intensity in the direction of greatest change at (i, j) , $y_{i,j}$ is an estimate of the between-rows rate-of-change in image intensity and $x_{i,j}$ estimates the between-columns rate-of-change:

$$y_{i,j} = \frac{1}{6} \begin{pmatrix} +f_{i-1,j-1} & +f_{i-1,j} & +f_{i-1,j+1} \\ \cdot & \cdot & \cdot \\ -f_{i+1,j-1} & -f_{i+1,j} & -f_{i+1,j+1} \end{pmatrix}$$

and

$$x_{i,j} = \frac{1}{6} \begin{pmatrix} +f_{i-1,j-1} & \cdot & -f_{i-1,j+1} \\ +f_{i,j-1} & \cdot & -f_{i,j+1} \\ +f_{i+1,j-1} & \cdot & -f_{i+1,j+1} \end{pmatrix}$$

Fig 2 shows the filter outputs for the three images in Fig 1.

Note that, because of differences in absorbance, refractility or optical thickness between cells, interference from underlying or overlying cells or because of intracellular structures, not all of the edges apparent in one image, may be observed in images from a different modality. For example, some algal cells in the brightfield image (Fig 2(a)) show edges, where there is a colour change at the edge of the chloroplasts, which are not apparent in the DIC image (Fig 2(c)). In turn, some cells in the DIC image show edges which correspond to vacuolar margins. In the phase contrast image many of the edges appear double because of the phase contrast halo artefact. However, we have not encountered any problems through these differences, and do not anticipate any in future provided such structures do not dominate the images in question.

We tried aligning our microscope images by applying both cross-correlation and phase-correlation criteria to gradient images. Because the cross-correlations performed slightly better, and also because there does not seem to be any theoretical justification for using phase-correlation with gradient images, we will only present results based on cross-correlations. Table 1 shows the maximum cross-correlation between pairs of the three gradient images in Fig 2, for each of a range of values of c . The largest value in each column is underlined. For each choice of c , a SPARCstation IPX took 5 minutes of CPU time using not particularly efficient Fortran77 code to compute all the cross-correlations among three images, each 512×768 pixels in size.

Table 1: Maximum % cross-correlation between pairs of gradient images for a range of values of c .

c	brightfield and phase contrast	brightfield and DIC	DIC and phase contrast
0.95		35.8	
0.96		38.3	
0.97		41.9	
0.98		45.9	
0.99		52.3	
1.00	17.2	<u>58.3</u>	19.2
1.01	17.1	53.5	20.6
1.02	17.9	46.2	22.0
1.03	19.5	41.6	22.6
1.04	21.2	38.7	25.0
1.05	22.6	36.4	27.3
1.06	<u>23.3</u>		<u>27.7</u>
1.07	21.9		26.4
1.08	19.9		23.9
1.09	18.4		22.8
1.10	17.6		21.9

These results are consistent, and indicate that the brightfield and DIC images are at the same magnification, whereas the phase contrast image has been magnified by a factor of 1.06. Fig 3 shows the arrays of cross-correlations displayed as intensity images for these selected magnifications. Larger values are displayed as darker. The location parameters which correspond to the maximum cross-correlations are given in Table 2, together with the estimated values of c .

Table 2: Estimated parameter values to align algal images

Images	i_0	j_0	c
brightfield and phase contrast	29	167	1.06
brightfield and DIC	2	5	1.00
DIC and phase contrast	29	164	1.06

These estimated translation parameters are close to, but not precisely, consistent: consistency would require those for the third pairing to be $i_0 = 27$ and $j_0 = 162$ given the first two sets of values.

The other two images can be transformed to the same scale as the brightfield image,

simply by assigning pixel (i', j') the value at (i, j) , where

$$i = \frac{i'}{c} - i_0, \quad \text{and} \quad j = \frac{j'}{c} - j_0.$$

However, if i' and j' are integers then in general i and j will not be. They could be rounded to the nearest integers, but it is preferable to use bilinear interpolation instead, with

$$f'_{i',j'} = ([i] + 1 - i)([j] + 1 - j)f'_{[i],[j]} + (i - [i])([j] + 1 - j)f'_{[i+1],[j]} \\ + ([i] + 1 - i)(j - [j])f'_{[i],[j+1]} + (i - [i])(j - [j])f'_{[i+1],[j+1]} \quad .$$

where $[.]$ is used to denote the integer part of a number.

To assess the robustness and accuracy of the alignment algorithm, the aligned images were divided into four quadrants which were then independently realigned. Rather than present estimated values of i_0 , j_0 and c , which can be difficult to interpret when $c \neq 1$, Table 3 instead gives the misalignment of the first row of each quadrant of the brightfield image with the first row of the corresponding quadrant in the other two images. For example, when the top-left quadrants of the brightfield and phase contrast images are aligned, the first row in the brightfield quadrant is misaligned by -1 , that is, one pixel width with respect to the first row in the phase contrast quadrant (recall that the images are 512×768 pixels in size). Table 3 also shows the misalignments of the last row and first and last columns of each quadrant. Misalignment is never more than 5 pixels on the borders of the quadrants, and will therefore be less than 5 pixels inside the quadrants. Overall, the root-mean-square misalignment is 2.5 pixels, which shows the alignment algorithm works well, in spite of the individual quadrants having far less information upon which to base their choices of magnification and translation parameters.

Table 3: Errors in alignment when the algorithm is applied to the four quadrants of aligned algal images

quadrant	first row	last row	first column	last column
brightfield and phase contrast				
1	-1	2	-3	1
2	2	-1	3	-1
3	1	-4	5	-3
4	0	-5	4	-4
brightfield and DIC				
1	2	-1	3	-1
2	2	-1	2	2
3	0	3	-3	1
4	0	-3	2	-2

3 Multimodal microscopy

Correlations between images, for the areas of overlap, were calculated on a pixel by pixel basis. These are given in Table 4.

Table 4: Correlations between algal images after alignment

	brightfield	phase contrast
phase contrast	-0.42	
DIC	0.90	-0.46

It can be seen that the brightfield and DIC images show the strongest association, with a correlation coefficient of 0.90. The phase contrast image is negatively correlated with the other two modalities, as is evident also in Fig 1: in (b) the cells are lighter than the background, whereas in (a) and (c) they are darker, on average. This is an optical consequence of the algal cells being relatively large and refractile. The resultant increased phase difference between the direct and the diffracted light beams leads to constructive interference in the image plane and hence bright instead of dark cells. The cluttered background in Fig 1(b) suggests that the poorer correlation coefficient for the phase contrast image probably results from the greater sensitivity of this imaging mode to interference from out of focus material.

3.1 Principal components analysis

Principal components analysis is one way of interpreting the correlation matrix. The first principal component is the linear combination of the images (after they have been standardized to have a common variance) which has maximum variability. The second principal component has maximum variability among linear combinations which are uncorrelated with the first principal component, and so on. (See, for example, Krzanowski (1988).) In image analysis applications, Savoiji and Burge (1985) have shown principal components to be efficient for feature extraction, image compression and noise filtering. Turk and Pentland (1991) used principal components of images of human faces for recognition. The three principal components of the algal image are given in Table 5.

Table 5: Principal components of aligned algal images

Component	1	2	3
% of variation	74	23	3
Weighting			
brightfield	0.62	0.35	-0.70
phase contrast	-0.46	0.89	0.04
DIC	0.63	0.29	0.72

The first principal component is an average of the brightfield and DIC images, with a smaller, negative contribution from the phase contrast image (Fig 4(a)). This accounts for 74% of the variability in the multimodal image. It strongly highlights the pigmented algal cells. The second principal component is essentially the phase contrast image, and explains 23% of the variability (Fig 4(b)). Phase-dark cells show improved contrast as compared with the original phase contrast image (Fig 1(b)). The final principal component is a difference between the DIC and brightfield images, which is discussed in §3.2. This is shown in Fig 4(c). Note the black borders at the top and left sides of these figures, produced by the alignment of images. The black squares in Fig 4(c) indicate subimages to be considered further.

Fig 5 is a pseudocoloured composite image, formed by arbitrarily assigning red to the first principal component, green to the second one and blue to the third one. By assigning colours to the principal components, rather than to the original images, a greater spread of colours is produced and more information can be extracted. Other criteria could have been used. For example, Green *et al* (1986) proposed a maximum noise fraction technique, in which a sequence of linear combinations of variables is found with noise levels decreasing through the sequence, whereas Nason and Sibson (1991) used projection pursuit to find interesting projections of multidimensional satellite data. The composite image reveals structural details in the optically-thick algal cells shown in the brightfield and DIC images (Figs 1(a), (c)) while also demonstrating optically-thin, phase-dark non-photosynthetic organisms (examples are shown as pinkish regions in the outlined rectangle in Fig 5). The image also clearly retains the distinction between the pigmented photosynthetic organisms, which appear dark, and non-photosynthetic organisms, which are coloured pinkish.

3.2 Construction of an image of optical thickness

If an object is completely transparent then DIC microscopy produces an image of the rate of change in a fixed direction of its optical thickness (Holmes and Levy,

1987, 1988). For objects such as the algal cells, which are only semi-transparent, the DIC image will be the sum of this rate of change and of the brightfield image. Therefore, the third principal component, in subtracting the brightfield image from DIC, recovers the rate of change image. If this is integrated along the same direction, then (in theory) an image of optical thickness can be constructed. In practice, this only seems to be achievable for small subimages and the sums need rescaling so that the background values are zero.

Consider an n by n subimage, such as that shown in the smaller black box in Fig 4(c). The direction of DIC is from top-left to bottom-right, so we will carry out the integration along diagonals. For all values of i between 1 and n , and all values of j between 1 and n , the constructed image $h_{i,j}$ is obtained as follows. If $i \leq j$, then

$$h_{i,j} = \sum_{k=0}^{i-1} f_{i-k,j-k} - \frac{i}{n+i-j} \sum_{k=j-n}^{i-1} f_{i-k,j-k} ,$$

otherwise

$$h_{i,j} = \sum_{k=0}^{j-1} f_{i-k,j-k} - \frac{j}{n-i+j} \sum_{k=i-n}^{j-1} f_{i-k,j-k} ,$$

where $f_{i,j}$ is a pixel in the third principal component. The second term in both expressions is there to ensure that the cumulative sums are zero at the borders of the image. The result is shown in Fig 6(d). For comparison, Figs 6(a)–(c) show the same pair of cells in brightfield, phase contrast and DIC microscopy.

To illustrate what happens to the reconstruction for larger subimages, Fig 7 shows the results of applying the same algorithm to the grouping of cells in the larger black box in Fig 4(c). Although an image of optical thickness can be discerned, diagonal streaking effects dominate the image. They are the result of speckle noise having a cumulative effect along each summed diagonal. The results are even worse if the whole image is analysed at once. Therefore, the best that can be achieved at present is to reconstruct isolated cells and pairs of cells.

4 Discussion

There is great potential for further development of image processing algorithms which exploit the information contained in light microscope images. Recombination of images obtained with different imaging modes is but one application. The alignment algorithm presented in this paper could also be used to align images of a three-dimensional specimen at a series of focal planes. Pseudocolour is another option available in digital image processing. Key features demonstrated in one

mode can be superimposed on images obtained with another. This would enable workers rapidly to visualise the structural environment of a particular feature, such as cells expressing a particular gene in studies like those of Modrusan *et al* (1994) or the precise locations of the labelled embryonic cells investigated by Collazo *et al* (1994). Many similar examples suggest themselves.

Multimodal microscopy offers opportunities for eliciting information which is unavailable from any single modality. For example, to estimate cell volumes it is necessary to know the vertical as well as the horizontal profile of the cell. The removal of attenuation components in the DIC image of a light absorbing cell such as the algal cells shown in this paper makes an image of the true optical profile of the cell accessible without recourse to a specialised interference microscope. (We have assumed that the whole cell is sufficiently in focus here to be involved in the imaging process. If this is not the case, then an overlying focal plane image may need to be incorporated.) In theory, the volume of a cell is directly proportional to the integrated intensity of the optical thickness image. Suitably calibrated, this would greatly facilitate volume measurement for cells with cross-sections which are not figures of rotation or for highly irregular cells such as the trypanosomes whose volumes were estimated by Webster and Griffiths (1994). It should be stressed however that practical verification of this proposal has not yet been undertaken and is dependent on further improvement in the image analysis to remove background artefacts.

Another example of the potential of multimodal microscopy is in the estimation of cell biomasses, for which it is necessary to know both the cell density and volume. Phase contrast and DIC images contain information on the refractive index, directly related to the density, of the cell contents. Appropriate algorithms could be developed to extract this information. Also, by combining fluorescence images of algal cells, the chloroplasts can be shown separately from the cytoplasm, allowing chloroplast volumes to be evaluated independently of the total cell volume. In summary, multimodality greatly widens the scope of investigations which can be performed with the standard light microscope.

Acknowledgements

The work was supported by funds from the Scottish Office Agriculture and Fisheries Department.

References

- Armbrust, V.A., Ferris, P.J. and Goodenough, U.W. (1993). A mating type-linked gene cluster expressed in *Chlamydomonas* zygotes participates in the uniparental inheritance of the chloroplast genome. *Cell*, **74**, 801-811.
- Augustine, P.C., Watkins, K.L. and Danforth, H.D. (1992). Effect of monesin on ultrastructure and cellular invasion by the turkey coccidia *Eimeria adenoides* and *Eimeria meleagrimitis*. *Poultry Science*, **71**, 970-978.
- Bajcsy, R. and Kovacic, S. (1989). Multiresolution elastic matching. *Computer Vision, Graphics and Image Processing*, **46** 1-21.
- Bracegirdle, B. (1993). A new look at old slides. *Microscopy and Analysis*, **34**, 33-35.
- Bookstein, F.L. (1991). *Morphometric Tools for Landmark Data : Geometry and Biology*. Cambridge University Press, Cambridge.
- Canny, J. (1986). A computational approach to edge detection. *IEEE Transactions on Pattern Analysis and Machine Intelligence*, **6**, 679-698.
- Chicino, G., Bruno, A., Cevini, C., Gatti, S. and Scaglia, M. (1990). Rapid microscopy technique for detection of *Pneumocystis carinii* in fresh clinical specimens. *European Journal of Clinical Microbiology and Infectious Diseases*, **9**, 601-605.
- Colchester, A.C.F. and Hawkes, D.J. (eds.) (1991). Information Processing in Medical Imaging. *Proceeding of the 12th International Conference on Information Processing in Medical Imaging*, Springer-Verlag, Berlin.
- Collazo, A., Fraser, S.E. and Mabee, P.M. (1994). A dual embryonic origin for vertebrate mechanoreceptors. *Science*, **264**, 426-430.
- Farkas, D.L., Baxter, G., DeBiasio, R.L., Gough, A., Nederlof, M.A., Pane, D., Pane, J., Patek, D.R., Ryan, K.W. and Taylor, D.L. (1993). Multimode light microscopy and the dynamics of molecules, cells, and tissues. *Annual Review of Physiology*, **55**, 785-817.
- Galbraith, W. and Farkas, D.L. (1993). Remapping disparate images for coincidence. *Journal of Microscopy*, **172**, 163-176.
- Germida, J.J. (1984). Persistence of *Nosema locustae* spores in soil as determined by fluorescence microscopy. *Applied and Environmental Microbiology*, **47**, 313-318.

- Glasbey, C.A. and Horgan, G.W. (1995). *Image Analysis for the Biological Sciences*. Wiley, Chichester.
- Green, A.A., Berman, M., Switzer, P. and Craig, M.D. (1988). A transformation for ordering multispectral data in terms of image quality with implications for noise removal. *IEEE Transactions on Geoscience and Remote Sensing*, **26**, 65-74.
- Holmes, T.J. and Levy, W.J. (1987). Signal-processing characteristics of differential-interference-contrast microscopy. *Applied Optics*, **26**, 3929-3939.
- Holmes, T.J. and Levy, W.J. (1988). Signal-processing characteristics of differential-interference-contrast microscopy. 2: Noise considerations. *Applied Optics*, **27**, 1302-1309.
- Horgan, G.W., Creasey, A.M. and Fenton, B. (1992). Superimposing two-dimensional gels to study genetic variation in malaria parasites. *Electrophoresis*, **13**, 871-875.
- Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994). *Arabidopsis* homeotic gene APETELA3 ectopic expression: transcriptional and post transcriptional regulation determine floral organ identity. *Cell*, **76**, 703-716.
- Krzanowski, W.J. (1988). *Principles of Multivariate Analysis : A User's Perspective*. Clarendon, Oxford.
- Kuglin, C.D. and Hines, D.C. (1975). The phase correlation image alignment method. In *Proceedings of the IEEE 1975 International Conference on Cybernetics and Society*, 163-165.
- Leonardi, A.J., Blakistone, B.A. and Kyryk, S.W. (1990). Applications of microscopy in the paper industry: case histories of the Mead corporation. *Food Structure*, **9**, 203-213.
- Martin, N.J. and Fallowfield, H.J. (1989). Computer modelling of algal waste treatment systems. *Water Science and Technology*, **21**, 277-287.
- Modrusan, Z., Reiser, R., Feldmann, R.A., Fischer, R.L. and Haughn, G.W. (1994). Homeotic transformation of ovules into carpel-like structures in *Arabidopsis*. *Plant Cell*, **6**, 333 -349.
- Moshfeghi, M. (1991). Elastic matching of multimodality medical images, *Computer Vision, Graphics and Image Processing*, **53**, 271-282.
- Nason, G.P. and Sibson, R. (1991). Using projection pursuit in multispectral image

analysis. *Proceedings of the 23rd Symposium on the Interface: Computer Science and Statistics*, (ed. E.M. Keramidas), 579-582.

Richards, J.A. (1986). *Remote Sensing Digital Image Analysis: An Introduction*. Springer-Verlag, Berlin.

Ried, T., Baldini, A., Rand, T.C. and Ward, D.C. (1992). Simultaneous visualisation of seven different DNA probes by *in situ* hybridisation using combinatorial fluorescence and digital imaging microscopy. *Proceedings of the National Academy of Sciences, USA*, **89**, 1388-1392.

Rosenfeld, A. and Kak, A.C. (1982). *Digital Picture Processing (2nd edition)*. Academic Press, San Diego.

Savoji, M.H. and Burge, R.E. (1985). On different methods based on the Karhunen-Loeve expansion and used in image analysis. *Computer Vision, Graphics and Image Processing*, **29**, 259-269.

Steinholt, H.C., Chandler, J.E. and Tirado, V. (1991). Evaluating acrosome reaction steps with brightfield and Differential Interference Contrast Microscopy Techniques. *Journal of Dairy Science*, **74**, 3822-3826.

Tang, Y.T. and Suen, C.Y. (1993). Image transformation approach to nonlinear shape restoration. *IEEE Transactions on Systems, Man and Cybernetics*, **23**, 155-171.

Turk, M. and Pentland, A. (1991). Eigenfaces for recognition. *Journal of the Optical Society of America, Series A*, **4**, 519-524.

Webster, F. and Griffiths, G. (1994). A novel method for mean cell volume estimation. *Journal of Microscopy* **174**, 85-92.

Captions for figures

N.B. Images are at reduced resolution for ftp

Fig 1 Microscope images, using different modalities, of a sample of algal and bacterial cells from an algal pond: **(a)** brightfield microscopy, **(b)** phase contrast microscopy, **(c)** DIC microscopy.

Fig 2 Results of applying Prewitt's edge filter to the algal images in Fig 1. Larger values from the edge filter are displayed darker and zero values are displayed as white.

Fig 3 Cross-correlations between gradients of algal images shown in Fig 2, with larger positive values displayed darker and negative values displayed lighter: **(a)** brightfield and phase contrast (magnified by a factor of 1.06), **(b)** brightfield and DIC, **(c)** DIC and phase contrast (magnified by a factor of 1.06).

Fig 4 Principal components of algal image after alignment: **(a)** first principal component, **(b)** second principal component, **(c)** third principal component, with two squares to be considered further in Figs 6 and 7.

Fig 5 Pseudocoloured composite image, formed from Fig 4 by assigning red to the first principal component of the multimodal algal image, green to the second principal component and blue to the third principal component. The rectangle encloses some pinkish regions, which show optically-thin, phase-dark non-photosynthetic organisms.

Fig 6 Images of the pair of algal cells enclosed by the smaller black square in Fig 4(c), using different microscope modes: **(a)** brightfield microscopy, **(b)** phase contrast microscopy, **(c)** DIC microscopy, **(d)** image of optical depth produced by directional integration of Fig 4.

Fig 7 : Image of optical depth produced by directional integration of algal cells enclosed by the larger black square in Fig 4(c).











