Fluoro- Morphometry, adding Fluorimetry to an image processing system for bacterial morphometry

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9. Summary

It has been known for some time that the human intestines contain large numbers of bacteria which are beneficial to the host through various mechanisms. This intestinal microflora is still very much *terra incognita* to science. Our inability to simulate *in vivo* growth conditions in the laboratory results in a sketchy knowledge at best of its varied, and immensely numerous population. Why this complete ecosystem lives in apparent harmony with the host's immune system is still a mystery. The GRID image processing system, parts of which have been described in this thesis, has been designed for the express purpose of giving new insights into both the response of the microflora to medication, and the interaction between the flora and the immune system. By analysing bacteria, obtained directly from the faeces, rather than by culturing them, a far more direct view of the intestinal flora is obtained than was hitherto possible. Originally only morphological data were acquired. Though these did provide a rapid screening of the ecological state of the intestinal flora, little knowledge pertaining to the actual contents of the flora in terms of bacterial species was obtained. For this reason, and because the interaction of the immune system with the flora could be assessed with the immunofluorescence technique, fluorimetry data were added. The scientific goals and history of the GRID system are reviewed in chapter 2.

When the addition of fluorimetry to the morphological package developed by Meijer and co-workers was first contemplated, the chief of the problems was thought to be the acquisition of the data. The combination of phase contrast or brightfield images and fluorescence posed a challenge to the camera system. Most sensitive cameras (such as slow scan CCDs and image intensifiers) are unable to cope with the large amount of light in the former type of image, whereas most video rate cameras are either not sensitive or not stable enough for fluorimetry. Nonetheless, video rate use of the Fairchild Weston CCD-5000/1 camera in the first implementation of the fluorimetry package, already allowed us to quantify immunofluorescence at a higher accuracy than with visual estimation. Chapter 3 introduces a long exposure board, which controls the integration time of the CCD 5000/1. It is shown that this has improved the sensitivity of the system by at least one order of magnitude, whilst retaining the option of video rate operation. The sensitivity of this new set-up is such that it can measure the faint autofluorescence of bacteria.

Once the basics of image acquisition had been solved, the restoration and calibration of the fluorescence images had to be addressed. The adoption of a moderately cooled CCD camera designed for video rate work, and using it in a long integration mode, increased the noise caused by dark current. Fortunately, this dark current noise is not entirely random, so that simple subtraction of this dark current eliminated most of this effect. Shading correction and calibration could be performed simultaneously using images of a solid fluorescence reference. To prevent noise in the dark current and shading images from degrading the fluorimetry data, a number of simple measures was taken. Firstly, summing N of reference images and dividing them by N, reduced the noise in the reference images. For optimum choices of N=4 for dark current and N=32 for shading, a reduction in noise of almost a factor of 2 has been achieved. Secondly, entire fluorescence images were *not* corrected for shading and stored in 8 bits per pixel. Instead, during analysis, quantitative fluorescence data were obtained by dividing the sum of grey levels over each area of interest by the sum of grey levels in the corresponding area in the shading image. This procedure reduces the propagation of round-off errors, resulting in 40% less noise. These technical issues are dealt

with at some length in chapters 4 and 5.
Chapter 6 shows "the proof of the pudding". The accuracy of the fluorimetry section of the image processing system was tested by measuring titres of serum antibodies directed against the intestinal flora in healthy volunteers. It was shown that titres could be measured to within 6 to 10 percent accuracy. This accuracy is some 20 times better than the first measurements using our system, and 35 times better than errors made by a single human observer. In the same trial, it was shown that longitudinal (biological) variations in the serum titres could be detected easily with this accuracy. It is therefore unlikely that any further improvement in sensitivity of the camera system would yield an increased sensitivity to medically or biologically significant changes in serum titres.
In any scientific environment where a large data bulk is acquired on a routine basis, graphical display facilities geared to explorative statistical analysis are essential. The DATAPLOT program, which was developed for such a purpose, is described in chapter 7. It introduces the morphogram, which shows the actual bacterial shapes at the appropriate locations in data space. By itself it gives insight into the types of objects present in various parts of the population. Combined with contour plots of bivariate density estimates, it allows the user to assess both morphology and relative numbers of objects. Univariate and bivariate density estimates of the populations computed with the adaptive kernel method are shown to be far superior to the histogram approach used in early implementations of the program.
As we are using low cost image processing systems based on personal computers, storage space is rather limited. Especially in a highly automated system, where large numbers of images are processed daily (100-300 per workstation), data compression is required. It has been shown in chapter 8, that a very simple algorithm, based on run length encoding (RLE), is highly effective in compressing our binary images containing morphological information. On images of full fields of view, compression ratios of more than 10 were realized, some 8% better than competing algorithms such as Lempel-Ziv and Welch, and modified Huffman RLE. In images of single areas of interest, several tens of thousands of which are produced each day, compression ratios of more than 600 were achieved. The other algorithms tested achieved between 30 and 50 times compression.