Automation of Fluorescent Dot Counting in Cell Nuclei

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Abstract

We have developed a completely automated fluorescence microscope system that can examine 500 cells in approximately 20 minutes to determine the number of labeled chromosomes (seen as dots) in each cell nucleus. This system works with two fluorescent dyes – one for the DNA hybridization dots (e.g. FITC) and one for the cell nucleus (e.g. DAPI). After the stage has moved to a new field the image is automatically focused, acquired by a Photometrics KAF 1400 camera, and then analyzed on a Macintosh Quadra 840AV computer. After the required number of cells has been analyzed, the user may interact to correct the computer by working with a gallery of the cell images. The machine accuracies are equal to panels of human experts (manual) and limited (ultimately) by the overlapping of dots in the 3D cell as seen through the 2D projection.

1: Introduction

Modern molecular technology has made it possible to selectively stain various DNA sequences in biological cells. These sequences may be chosen so as to detect specific abnormalities (e.g. Tay–Sachs, Huntington's) or to facilitate the process of counting chromosome types. This latter possibility is especially important in pre-natal counseling. By labeling human chromosome 21, for example, it becomes possible to identify three chromosomes (trisomy) in the cell instead of the normal two chromosomes. This particular aberration is associated with Down's syndrome. The labeling process reveals specific chromosomes as dots in the (interphase) cell nucleus.

A variety of staining mechanisms have been described for this technique (called *in situ* hybridization) including labeling with radioactive isotopes or using light absorbing dyes. The most exciting and promising procedures, however, are based upon the use of fluorescent dyes. These dyes are attached to the DNA probes and when excited by one wavelength of light emit light at a second, longer, wavelength. Typical fluorescent dyes are described in Table 1.

Dye	Emission Wavelength	Use
DAPI	452 nm.	Make nucleus visible
FITC	523 nm.	Detect DNA sequenc
Texas Red	615 nm.	Detect DNA sequenc

Table 1: Typical fluorescent dyes used in FISH – fluorescent *in situ* hybridization

The major disadvantage associated with the use of fluorescent dyes is the weak signal emanating from a cell or its labeled constituents. A background pixel consists of typically 5000 photons, a DAPI-stained nucleus pixel is an additional 3000 photons, and a labeled chromosome pixel an additional 10000 photons. These numbers are at least a factor 100 smaller than the values that are obtained for images acquired through conventional CCD cameras at room-level illumination [1].

In a clinical setting it is necessary to review 500 to 1000 cells in order to determine the distribution of the number of dots per cell and to be able to detect small aberrant sub-populations of cells. Further, practical considerations require that such a test be performed in a short time (<20 minutes). Current manual counting procedures leave much to be desired including the need to work in darkened environments (because of the weak fluorescent signals) and the fatiguing nature of the work.

We have developed a completely automated microscope system that counts fluorescent hybridization spots for one probe in an interphase cell nuclei. Only 2 colors, such as DAPI and FITC, can be used – one for the stain to make the chromosome probe visible and one for the counterstain to make the entire nucleus visible. The images as observed through a fluorescent microscope are then passed through a special filter set to map the blue fluorescence into the

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middle gray values and the green values into the light gray values. A Photometrics (KAF 1400) high-sensitivity, low-noise cooled CCD camera is used to acquire a digitized image of 1320 x 1035 pixels with 4096 gray levels per pixel. Each image is integrated for 4.0 seconds to achieve images of suitable contrast and SNR. The SNR is limited only by the photon statistics which are themselves Poisson distributed. This means that the noise is not additive, not Gaussian, and not independent of the signal. A small portion of a typical image is shown in Figure 1.

Through the judicious use of the color filters the image processing is based on *brightness contrast* and not on color information. This is necessary because current technology does not – as yet – provide high resolution, high sensitivity, low-noise color cameras with three independent color channels.

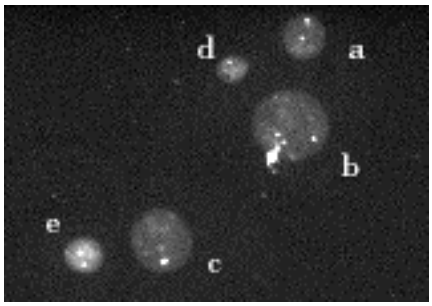

Figure 1: Double stained cells (DAPI + Spectrum Orange™). This subimage is 160 by 111 pixels sampled at 3.6 pixels per µm. **a)** 2 dots; **b)** 3 dots plus stain debris; **c)** 1 dot; **d)** 1 dot, and; **e)** 2 dots.

2: Procedure

A complete scanning cycle consists of automated microscope stage motion, automated focusing on a *binned* version of the fluorescent image, and then acquisition of the entire image. After acquisition the image is processed while the next stage motion takes place. This continues until a preset number of cells has been analyzed. The entire operation is under control of a Macintosh Quadra 840AV. The software has been written in Symantec Think C v6.0.

• Auto-focusing — A derivative filter [1,-1] is applied in the *y* direction of the image, *i*, and the "energy" in the resulting image is computed giving the focus function $F(z) = \sum_x \sum_y \{i(x,y,z) - i(x,y-1,z)\}^2$. $F(z)$ is to be maximized over z. The image *must* be sampled at *half* the Nyquist frequency in the filter direction. The sampling frequency in the other direction can be smaller. Binning (2x in *y* and 4x in *x*) is used to reduce the integration time, the readout time, and the processing time. The Nyquist frequency can be calculated from the formula $f_N = 4NA/\lambda$ where *NA* is the numerical aperture of the microscope lens and λ the wavelength of fluorescence *emission*. The focus function is first sampled with a step size of 3 µm to localize the maximum. Around the peak a step size of 0.75 µm is used. Focus samples around the peak are used to fit a quadratic function. This fit gives the position of the maximum focus [2].

• Image analysis — Our algorithm for actually counting the number of dots per cell nucleus can be divided into four steps: 1) Find a region that contains a nucleus (ROI), 2) Find the nucleus in the ROI, 3) Find the spots in the nucleus, and, 4) Count the spots and make (update) a histogram for the entire microscope slide.

Step 1 — To speed-up the algorithm the original image is subsampled by a factor of 8. After a shading correction based upon a gray-level opening, the image is segmented by a constant threshold [3]. Because only a small percentage of the area contains cells, the first step significantly reduces the amount of data.

Step 2 — For each ROI the original image is "re-segmented" using the Isodata thresholding algorithm [4] . Using morphological operations on the binary image, small objects are removed and slightly touching objects are separated [5] . Size, intensity, and shape features are measured for each object [6]. These features are used to make a distinction between real single nuclei, debris, touching nuclei, etc.

Step 3 — A threshold is performed on a TopHat Transform of the original image to find the spots [7]. Most spots are detected properly with the TopHat transform, but some spots are merged. Therefore a nonlinear Laplacian (nL) is performed on the TopHat image [8]. A threshold at a negative level will separate the spots (see Figure 2). The binary image of the TopHat transform and nL are combined. Once again, features of the putative dots (intensity and size) are measured to refine the procedure.

Step 4 — The spots are counted and the final result of the spot counter algorithm can be interactively verified and corrected. The individual nuclei are automatically relocated under the microscope. Visual inspection can be done using the monitor display or through the microscope.

The output of dot counting in interphase nuclei can be a confusion matrix, a dot histogram, and/or a gallery of images of *every* cell that has been analyzed. The preferred form is the dot histogram which gives the number of cells containing 0, 1, 2, 3, 4, or >4 dots. The uncertainty associated with the number of dots counted in the six "bins" {0,1,2,3,4,>4} leads to a *multinomial* distribution where the sample mean (m) and sample standard deviation (s) are given by $m = Np_i$ and $s^2 = Np_i(1-p_i)$.

in: Proc. 12th IAPR International Conference on Pattern Recognition, Jerusalem, Israel, 1994, 84-87.

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easily be increased by another factor of 2. The user can review and correct the data in a rapid manner (currently <5 minutes) through the use of galleries. (See Figure 3 below.) Finally, fundamental limits to accuracy are based on the size of the dots, the cells, and the numerical aperture of the microscope lens.

5: References

[1] Mullikin JC, et al., Methods for CCD Camera Characterization, in *SPIE Proceedings Image Acquisition and Scientific Imaging Systems*, San Jose, California, 1994, Vol. TC-2173.

[2] Van Vliet LJ, Boddeke FR, De Jong P, Netten H, and Young IT, Autofocusing in Fluorescence Microscopy, in *Proceedings XV Congress of the International Society for Analytical Cytology*, Bergen, Norway, 1991, Cytometry, Supplement 5, pp. 70.

[3] Zack GW, Spriet JA, Latt SA, Granlund GH, and Young IT, Automatic detection and localization of sister chromatid exchanges, *Journal of Histochemistry and Cytochemistry*, **24**(1), 1976, 168-177.

[4] Ridler TW and Calvard S, Picture thresholding using an iterative selection method, *IEEE Transactions on Systems, Man, and Cybernetics*, **SMC-8**(8), 1978, 630-632.

[5] Giardina CR and Dougherty ER, *Morphological Methods in Image and Signal Processing*, Prentice–Hall, Englewood Cliffs, New Jersey, 1988.

[6] Young IT and Roos R, Acuity: Image analysis for the personal computer, in *Pattern Recognition and Artificial Intelligence* (Gelsema ES and Kanal LN, Ed.), pp. 5–16, Elsevier Science Publishers, Amsterdam, 1988.

[7] Meyer F, Iterative Image Transformations for an Automatic Screening of Cervical Cancer, *Journal of Histochemistry and Cytochemistry*, **27**, 1979, 128-135.

[8] Van Vliet LJ, Young IT, and Beckers ALD, A Non-linear Laplace Operator as Edge Detector in Noisy Images, *Computer Vision, Graphics, and Image Processing*, **45**, 1989, 167-195.

[9] Van Vliet LJ, Young IT, Prins M, and Netten H, *Hiding dots in spherical and oblate objects*, Internal memorandum, Pattern Recognition Group, Faculty of Applied Physics, Delft University of Technology, 1993.

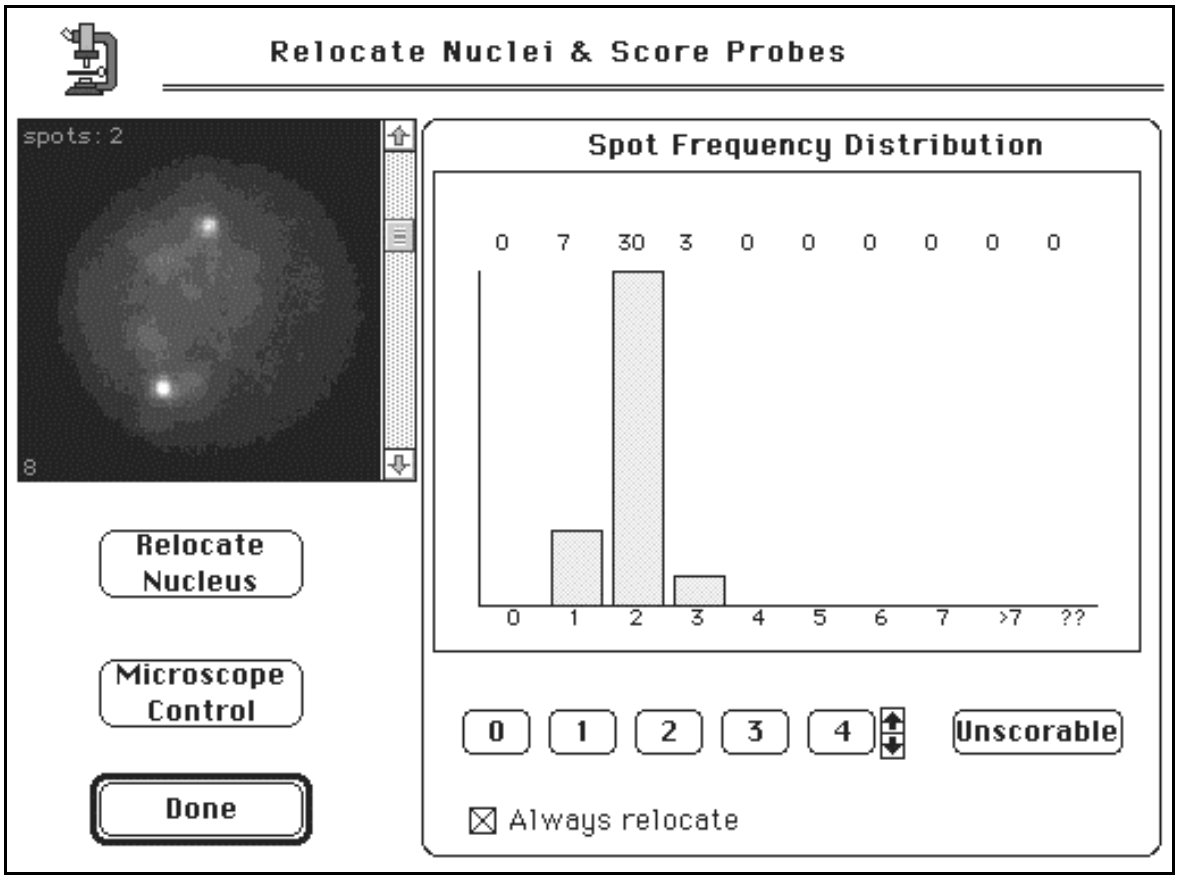

Figure 3: Example of the interactive dialog window that is used to manually correct one or more cells that have been selected from the gallery (not shown.) "Relocation" refers to an automatic translation of the microscope table to the cell nucleus if the microscope slide is under the lens.

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