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Confocal 3D DNA cytometry: assessment of required coefficient of variation by computer simulation [1]

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Abstract. *Background*: Confocal Laser Scanning Microscopy (CLSM) provides the opportunity to perform 3D DNA content measurements on intact cells in thick histological sections. So far, sample size has been limited by the time consuming nature of the technology. Since the power of DNA histograms to resolve different stemlines depends on both the sample size and the coefficient of variation (CV) of histogram peaks, interpretation of 3D CLSM DNA histograms might be hampered by both a small sample size and a large CV. The aim of this study was to analyze the required CV for 3D CLSM DNA histograms given a realistic sample size. *Methods*: By computer simulation, virtual histograms were composed for sample sizes of 20000, 10000, 5000, 1000, and 273 cells and CVs of 30, 25, 20, 15, 10 and 5%. By visual inspection, the histogram quality with respect to resolution of G0/1 and G2/M peaks of a diploid stemline was assessed. *Results*: As expected, the interpretability of DNA histograms deteriorated with decreasing sample sizes and higher CVs. For CVs of 15% and lower, a clearly bimodal peak pattern with well distinguishable G0/1 and G2/M peaks were still seen at a sample size of 273 cells, which is our current average sample size with 3D CLSM DNA cytometry. *Conclusions*: For unambiguous interpretation of DNA histograms obtained using 3D CLSM, a CV of at most 15% is tolerable at currently achievable sample sizes. To resolve smaller near diploid stemlines, a CV of 10% or better should be aimed at. With currently available 3D imaging technology, this CV is achievable.

Keywords: Confocal laser scanning microscopy, DNA ploidy, histogram quality, sample size, computer simulation

1. Introduction

Confocal Laser Scanning Microscopy (CLSM) presents the opportunity to perform 3D DNA content measurements on intact cells in thick histological sections [12]. This has major advantages over flow cytometry and image cytometry on cytospins which requires dissociation of tissue with consequent loss of tissue architecture, as well as over conventional image cytometry on thin tissue sections which suffers from cutting artefacts or overlap [13]. So far, sample size has been limited by the time consuming nature of the technology. Therefore, much attention has been paid to automation in order to arrive at acceptable sample sizes [2,9,10]. Still, sample sizes that have been achieved even with automated 3D CLSM are modest and in practice do not exceed 300 cells [5], nowhere near the sample size of 1,000 cells easily achieved with automated conventional 2D image cytometry [1,6] or the over 40,000 cells measured in minutes by flow cytometry [4,11].

Since the power of DNA histograms to resolve different stemlines depends on both the sample size and the coefficient of variation (CV) of histogram peaks [7], the relatively modest sample size achieved with 3D CLSM might hamper interpretation of the result-

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ing DNA histograms. This could be compensated by optimal imaging technology that keeps CVs low. The aim of this study was to analyze by computer simulation the required CV for 3D CLSM DNA histograms to separate G0/1 and G2/M peaks given a realistic sample size. The ability to separate the peaks is important when the linearity of the measurement system is established.

2. Materials and methods

By computer simulation, histograms were composed from normally distributed G0/1 and G2/M peaks of a diploid stemline for sample sizes of 20000, 10000, 5000, 1000, and 273 cells (the latter being our current average sample size with 3D CLSM DNA cytometry) and CVs of 30, 25, 20, 15, 10 and 5%. CVs of G0/G1 and G2/M peaks increased simultaneously. Random samples were drawn using the random number generator in Microsoft Excel. The ratio of the nuclei in the G2/M phase to all nuclei was set to 0.06, i.e., 6% of the cell population was expected to be in the G2/M phase of the cell cycle. The mean value of the G0/1 stemline was set at 100 (a.u.) and the mean value of the G2/M stemline at 200. The randomly generated values were divided into discrete bins with a width of a single arbitrary unit. By visual inspection, the histogram quality with respect to resolution of G0/1 and G2/M peaks was assessed. Furthermore, a receiver operating characteristic (ROC) analysis was performed. For all simulated populations the specificity and the sensitivity were assessed and the area under the ROC curves was computed.

3. Results

Figures 1–3 show the histograms of the different simulations. As expected, the interpretability of DNA histograms deteriorated with decreasing sample sizes and higher CVs. For CVs of 5% and 10%, the histograms hardly seemed to deteriorate with decreasing sample size, and the width of the G0/1 peak would allow resolution of smaller near-diploid aneuploid stemlines. For a CV of 15%, a clearly bimodal peak pattern with well distinguishable G0/1 and G2/M peaks was still seen at a sample size of 273, but it was clear that it would be difficult to resolve smaller near-diploid aneuploid stemlines. For CVs higher than 15%, the bimodal peak pattern was progressively lost with smaller samples sizes and even bigger triploid additional stemlines would be hard to resolve.

In Fig. 4 the result of ROC analysis is shown. For CVs up to 10% the area was 1, because the distinction between the G0/1 peak and G2/M peak was unambiguous. For histograms corresponding with CVs of 15% and larger, the area under the curve reduces as a result of amalgamation of both peaks. The area under the curve depends on the sample size and is noisy for a small number of nuclei.

4. Discussion

The aim of this study was to analyse by computer simulation the required CV for 3D CLSM DNA histograms to separate G0/1 and G2/M peaks given a realistic sample size. As expected, the interpretability of DNA histograms deteriorated with decreasing sample sizes and higher CVs. For CVs of 15% and lower, a clearly bimodal peak pattern with well distinguishable G0/1 and G2/M peaks were still seen at a sample size of 273, which is our current average sample size with 3D CLSM DNA cytometry. Other studies using 3D CLSM DNA cytometry employed sample sizes ranging from 22 to 248 and a CV of approximately 15% [8,15]. In other words, the required CV of 15% or less for CLSM measurements is only just satisfied. In a previous report, we showed that CVs smaller than 10% could be achieved on model tissue using 3D CLSM image cytometry, indicating that using the CLSM technique is indeed clinically feasible [14]. In real tumours, CVs of "diploid" peaks are however usually larger due to genetic instability with varying DNA content of these "diploid" cells. This superimposed CV increase may hamper detection of near-diploid aneuploid peaks, a problem encountered in DNA image and flow cytometry in general. Aiming at the lowest possible "system" CV remains therefore essential. This simulation study makes it possible to estimate the target CV almost effortlessly, whereas a thorough analysis of actual data from real tumour samples would take an enormous amount of time. Important issues to arrive at the lowest possible system CV include default settings and alignments provided by microscope manufacturers, refractive index mismatches and errors due to attenuation in depth, and stoichiometry of the staining reactions. Possible methods to improve the quality of confocal measurements should address the points raised in the document "Critical Aspects of Fluorescence

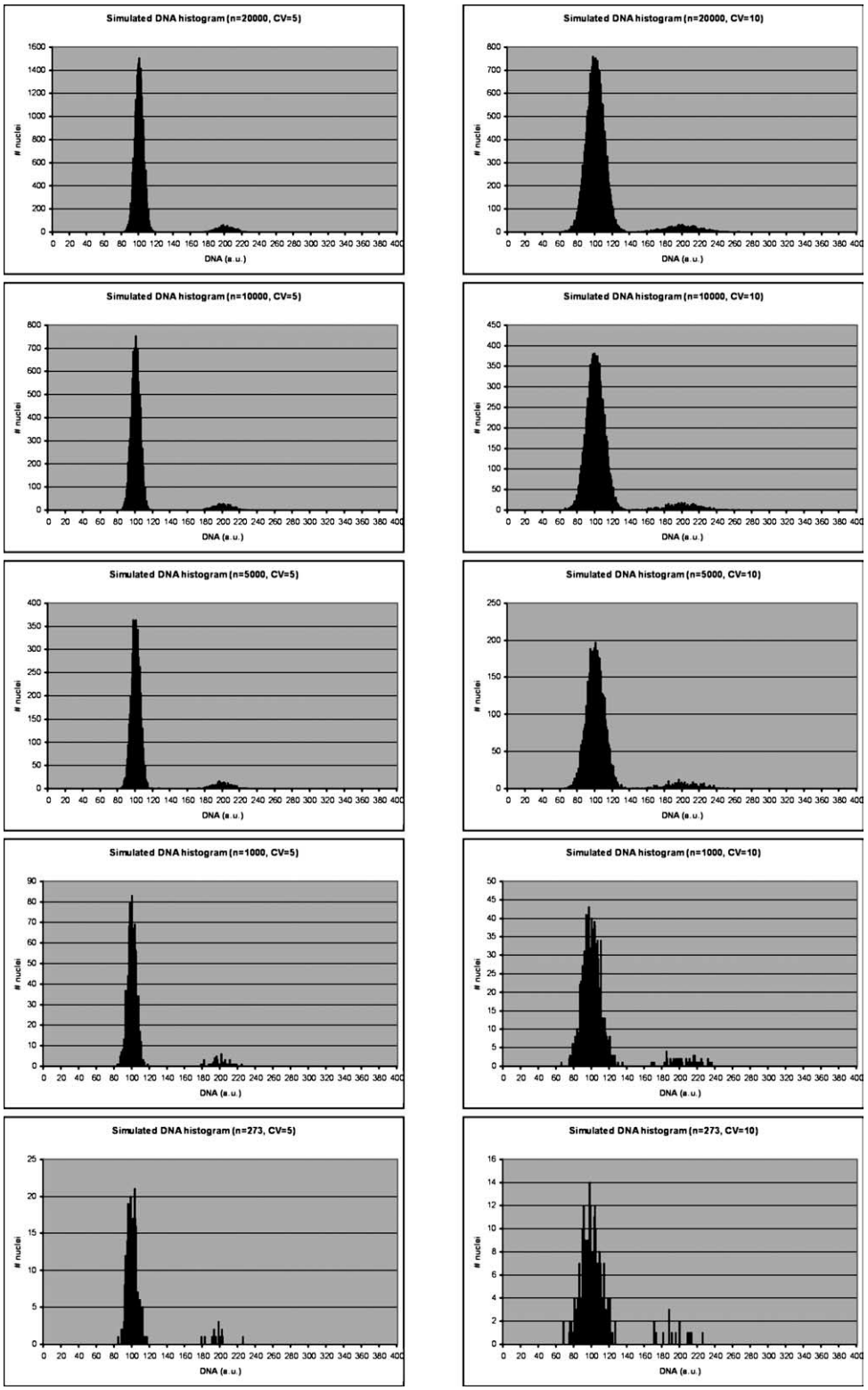

Fig. 1. Synthesized histograms with normally distributed G0/1 and G2/M peaks with a decreasing number of nuclei. The CV was set to 5 and 10%, respectively, while the number of nuclei gradually decreased from 20,000 to 273 (our current average sample size with 3D CLSM DNA cytometry). In all cases, the distinction between the G0/1 and G2/M phase is well defined.

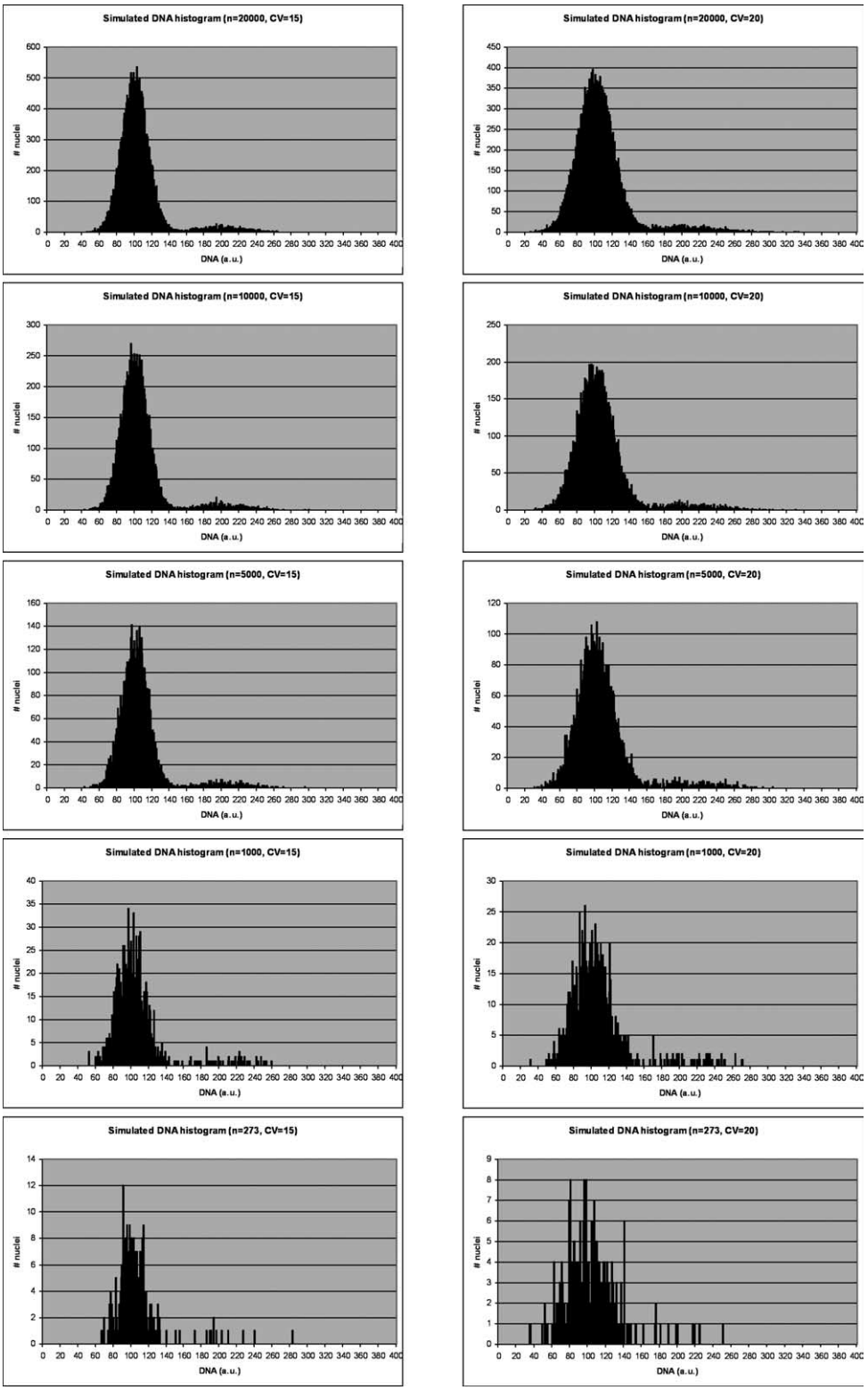

Fig. 2. In this series of histograms the CV was set to 15 and 20% and the number of nuclei gradually decreased as in Fig. 1. For a small number of nuclei and a CV of 20% the bimodal peak pattern was progressively lost.

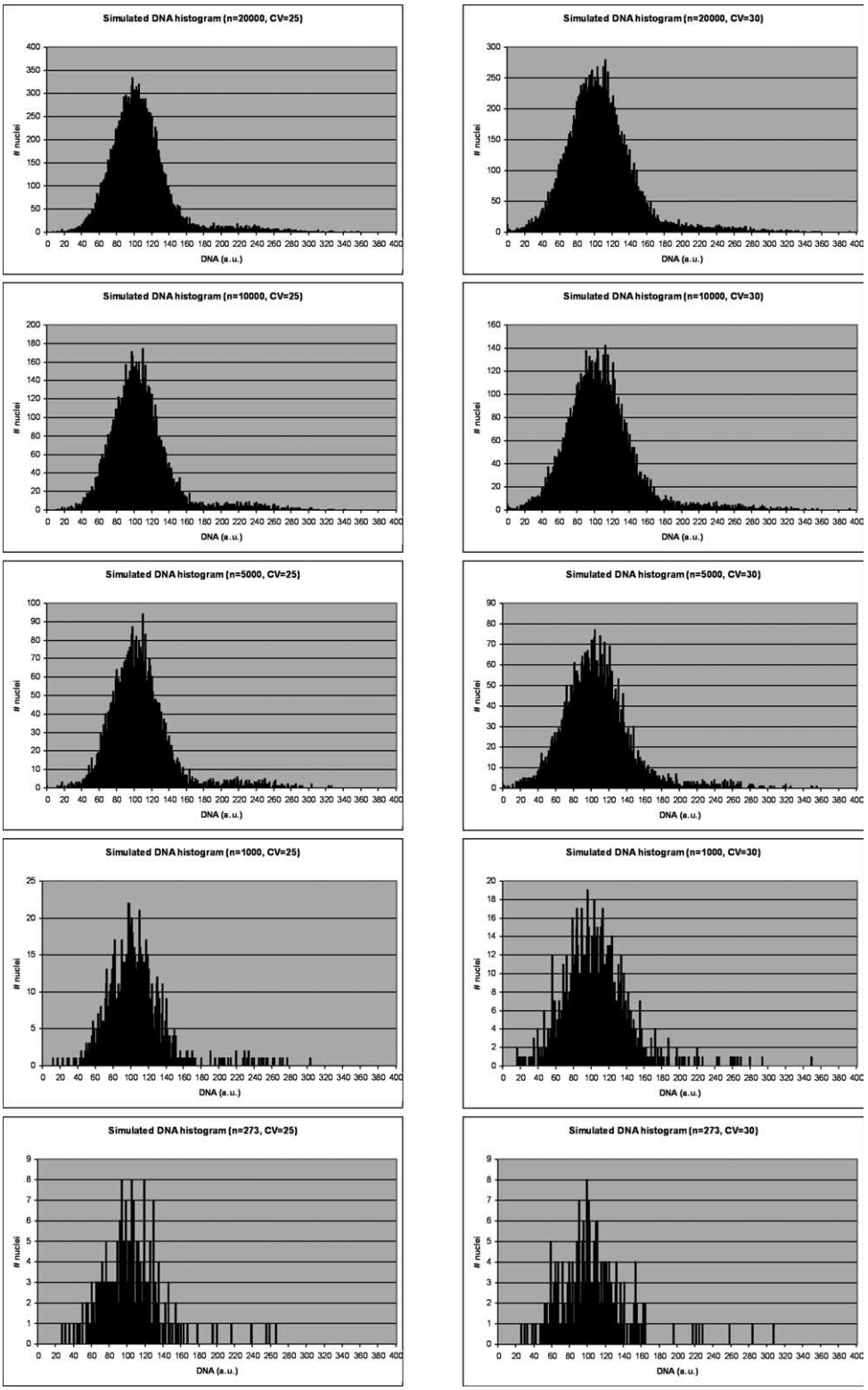

Fig. 3. In the last series of histograms corresponding with CVs of 25% and 30%, both peaks amalgamate and the location of the boundary between both peaks is ill defined, even when 20,000 nuclei are measured. This effect is most apparent for a CV of 30% where the G2/M peak appears as a shoulder of the G0/1 peak.

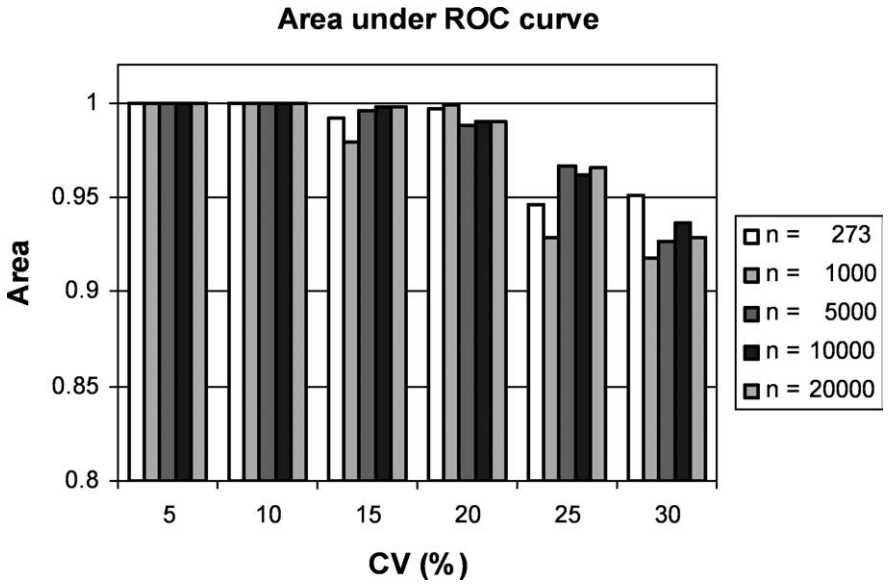

Fig. 4. Receiver operating characteristic (ROC) analysis for all simulations. The area under the curves is plotted. For CVs up to 10%, identifying the distinction between the G0/1 peak and G2/M peak is unquestionable. For histograms corresponding with CVs of 15% and larger, the area under the curve reduces, indicating amalgamation of both peaks. For a small number of nuclei, the determined area under the curve depends on the specific sample and hence is noisy.

Confocal Microscopy" (http://www.microscopyu.com/articles/confocal/pawley39steps.html).

The CV depends on both the quality of tissue preparation and on the machine performance. Currently, we are optimising the procedure for tissue preparation by investigating the influence of detergents and application of a post-fixative. Furthermore, reduction of inhomogeneous field illumination by applying a procedure for flat field correction to improve the CV currently is being investigated. This procedure removes a dependency on the location of the nuclei within each field. Other factors that influence the CV include laser stability, performance of the photo multiplier, system linearity, dynamic range, depth resolution, and lens quality. Each of these factors should be adjusted for optimal performance.

Concerning the small sample size, a higher level of automation might increase the number of measured nuclei to reach the 1,000 level. For this purpose, both acquisition of the image stacks and segmentation of the nuclei should be possible with a minimum of user interaction. Not many previous studies have addressed the problem of sample size and CV in DNA cytometry. We previously reported that for DNA flow cytometry, a sample size of 40,000 events was necessary for reliable combined determination of DNA ploidy, DNA index and %S-phase cells [4]. As far as we know, the relation between sample size and CV has not been previously reported for DNA image cytometry. However, the relation between CV, the location of aneuploid peaks and the percentage of non-diploid cells was studied by computer simulation by Benson et al. [3]. With the help of a chart the minimum CV required to resolve diploid and aneuploid peaks can be determined. It is not possible to estimate the required number of measurements with this graph, as this variable was not part of the model. In this study the ratio between the number of cells in the G2/M phase and the number of cells in the G0/1 phase was fixed to 0.06, in line with the average cycling cell population. Although this ratio may vary, especially in tumours, we believe that the impact of the ratio is small compared to the influence of the number of cells and the CV, e.g., even for a CV of 15% and only 273 cells, sufficient events are measured for this ratio to obtain a noticeable G2/M peak. Furthermore, it is likely that with an increasing number of cells in the G2/M phase the detectability will increase.

Progress in the field of confocal cytometry in the future will depend heavily on improvements to the speed of image acquisition. Multibeam and multiphoton techniques may contribute significantly to this effort, and practical spinning disk systems exist for live cell applications, with some compromises in axial resolution. However, confocal laser scanning remains the standard methodology since it presents the fewest com-

promises and best axial resolution. Histogram smoothing, which is a standard option in DNA histogram interpretation software, may be thought to improve the interpretability of ragged histograms based on small samples. However, for a CV of, e.g., 20%, a very broad kernel width of the smoothing filter would be required and as a result smoothing will actually increase the CV of the G0/1 peak.

In conclusion, for unambiguous interpretation of DNA histograms obtained using 3D CLSM, a CV of at most 15% is tolerable at currently achievable sample sizes. To resolve smaller near diploid stemlines, a CV of 10% or better should be aimed at.

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