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Strategies for Rare-Event Detection: An Approach for Automated Fetal Cell Detection in Maternal Blood

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Summary

This article explores the feasibility of the use of automated microscopy and image analysis to detect the presence of rare fetal nucleated red blood cells (NRBCs) circulating in maternal blood. The rationales for enrichment and for automated image analysis for "rare-event" detection are reviewed. We also describe the application of automated image analysis to 42 maternal blood samples, using a protocol consisting of one-step enrichment followed by immunocytochemical staining for fetal hemoglobin (HbF) and FISH for X- and Y-chromosomal sequences. Automated image analysis consisted of multimode microscopy and subsequent visual evaluation of image memories containing the selected objects. The FISH results were compared with the results of conventional karyotyping of the chorionic villi. By use of manual screening, 43% of the slides were found to be positive (≥1 NRBC), with a mean number of 11 NRBCs (*range* 1–40). By automated microscopy, 52% were positive, with on average 17 NRBCs (*range* 1–111). There was a good correlation between both manual and automated screening, but the NRBC yield from automated image analysis was found to be superior to that from manual screening ($P = .0443$), particularly when the NRBC count was >15. Seven (64%) of 11 XY fetuses were correctly diagnosed by FISH analysis of automatically detected cells, and all discrepancies were restricted to the lower cell-count range. We believe that automated microscopy and image analysis reduce the screening workload, are more sensitive than manual evaluation, and can be used to detect rare HbF-containing NRBCs in maternal blood.

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Introduction

Many researchers are currently exploring the possibility of performing noninvasive prenatal diagnosis by detecting fetal cells in maternal blood (Bianchi et al. 1990; Hamada et al. 1993; Lo et al. 1993; Zheng et al. 1993; Simpson and Elias 1994; Ganshirt et al. 1995; Cheung et al. 1996; Tanke et al. 1996). Fetal DNA sequences have been detected, by PCR, using Y-chromosome–specific primers, in maternal whole blood (Suzumori et al. 1992; Hamada et al. 1993; Langlois and Wilson 1993; Liou et al. 1993; Bjorkqvist et al. 1994; Lo et al. 1994; Thomas et al. 1995) and even in plasma and serum (Lo et al. 1997, 1998). However, for simultaneous recognition of intact fetal cells and the diagnosis of a molecular or chromosomal abnormality, the usual approach is to ascertain *specific* fetal cell populations, such as fetal nucleated red blood cells (NRBCs; Lo et al. 1994; Simpson et al. 1995; Bianchi et al. 1996; Cheung et al. 1996; Lewis et al. 1996; Sohda et al. 1997; Bianchi 1998).

Because fetal NRBCs are present in maternal blood at frequencies as low as $1/10^4$–$1/10^9$ nucleated maternal cells, dependent on methodology and gestational age (Bianchi et al. 1990, 1997; Hamada et al. 1993; Thomas et al. 1995), many enrichment strategies have been developed. At present, ficoll density gradient centrifugation procedures (Bhat et al. 1993), selective cell lysis (Saunders et al. 1997), flow sorting (Herzenberg et al. 1979; Lewis et al. 1996), magnetic-activated cell sorting (Ganshirt-Ahlert et al. 1992), and combinations thereof are used to enrich target cells or to deplete maternal background cells (e.g., erythrocytes and leukocytes).

Although enrichment does not actually increase the number of NRBCs, it does reduce the amount of maternal background cells. The rationale for enrichment is therefore both a technical and an economic one: the reduction of background cells may, on one hand, facilitate target-cell identification and, on the other hand, may decrease the number of slides that have to be screened to detect statistically sufficient numbers of target cells, thereby reducing the workload. Many steps in

the enrichment procedure are laborious, may affect target-cell morphology, and will inevitably lead to cell loss as a result of the procedure itself. Since a 100%-reliable single fetal NRBC marker is not available, none of the enrichment procedures is 100% specific (Bianchi 1995). This is illustrated by the frequent use of combinations of markers, such as the transferrin receptor, glycophorin A, and fetal hemoglobin. Thus, maximum depletion of (maternal) background cells may be associated with significant target-cell loss. Attempts to recover all target cells present in the original maternal blood sample can be achieved only by use of a less stringent enrichment, but this in turn results in a smaller reduction in the number of background cells.

In the reassessment of the rationale for enrichment, a different approach could be chosen, such as to optimize, accelerate, and economize the screening of slides by use of automated microscopy and automated image analysis. In this case, less stringent enrichment would be sufficient, the loss of NRBCs would be reduced, and a higher target-cell yield would be produced. One prerequisite, however, is that target-cell staining and recognition be unhampered by background material. To this end, a preparation and staining protocol has been developed (Oosterwijk et al. 1998*b*), which in a previous study proved to be suitable for fetal NRBC detection using manual screening (Oosterwijk et al. 1998*a*). On the basis of our experience with rare-event detection using automated image analysis (Verwoerd et al. 1987; Mesker et al. 1994; Ploem-Zaaijer et al. 1994), we performed a pilot study comparing manual NRBC detection with automated microscopy and analysis of multimode images, which allows the use of bright-field staining as well as fluorescent markers for specific identification of fetal cells (Tanke et al. 1996). The objective of the present study was to assess the applicability of automated microscopy and image analysis for the detection of fetal NRBCs in maternal blood. In this article, we present our results and discuss some applications.

The Concept of Rare-Event Detection

The screening of large numbers of cells in order to detect rare (abnormal) target cells occurs in various medical settings, such as in obstetrics, to detect fetomaternal transfusion (Cupp et al. 1984; Lloyd Evans et al. 1996); in oncology, to detect cytologically abnormal cells in cervical smears or to quantify residual disease (Mansi et al. 1988); in mutation research, to detect mutant erythrocytes containing hemoglobin S (Verwoerd et al. 1987); and in the early detection of cytomegalovirus reactivation in transplantation patients (Ploem-Zaaijer et al. 1994). In general, the detection of rare-event cells is facilitated by use of specific staining methods (e.g., monoclonal antibodies directed to defined cellular constituents or molecules of the cells of interest). Specific staining such as this increases the sensitivity and specificity of cell detection, unlike morphologic recognition alone, which is subject to bias and is less clearly defined. Visual screening of specifically stained slides is still a laborious task, however, and at low frequencies target cells can easily be missed. In addition, manual screening may be subject to inter- and intraobserver variability, which especially affects the accurate quantification of results.

For automated detection and quantification of rare-event cells, two main approaches can be distinguished: flow cytometry (Herzenberg et al. 1979; Lewis et al. 1996) and image cytometry (Ploem et al. 1986; Mesker et al. 1994; Tanke et al. 1996). Both methods rely heavily on the use of highly specific markers for cell recognition. Flow cytometry followed by cell sorting is an enrichment procedure, whereas image cytometry is not. Flow cytometry is superior in speed, but image cytometry offers the advantage that the image of each detected event can be stored in memory, for subsequent visual verification. Moreover, the detected cells can also be automatically relocated for direct microscopic inspection, since their positions on the slides are also recorded. An additional technical advantage of image cytometry is that some staining methods (e.g., FISH) are more suitable for cells on glass slides than for cells in suspension. Given the low frequency of fetal NRBCs and the necessity to preserve NRBC morphology, our method of choice was computerized microscopy and image analysis using an interactive approach. By such a method, candidate fetal NRBCs could be automatically detected, stored in the database, and presented to the operator for verification.

Automated Image Cytometry

Preparation

Automated microscopy performed on the basis of image-plane scanning using charge-coupled–device cameras is best accomplished at an optimal cell density. Too low a cell density will slow down the analysis process, because more microscopic fields have to be analyzed in order to examine the same number of cells. Too high a cell density will result in more cells that touch and overlap, which complicates the analysis, reduces target-cell recognition, and increases the number of artifacts. Automated microscopy uses automated focus control, which makes it necessary for the cells to be positioned on the slide in a single layer. The preparation of slides has to be of very high and reproducible quality. Because the automated microscope will scan a precisely defined region of the slide, the cells have to be fixed on the slide in exactly that region. All of these conditions are best met by the technique of centrifugal cytology (Driel-Kulker et al. 1980; Leif 1983; Ploem-Zaaijer et al. 1994),

which we recently adapted for application to NRBC detection (Oosterwijk et al. 1998*b*).

Target-Cell Identification

Cell recognition, whether manual or automated, is always performed on the basis of specific discriminating characteristics. For fetal NRBCs, we used (1) cell morphology, (2) the presence of a nucleus, and (3) the presence of HbF that has been immunocytochemically stained by monoclonal antibodies for the Hb gamma chain and visualized by bright-field microscopy (Oosterwijk et al. 1998*b*). This approach appears to be highly specific with manual screening and can be combined with FISH analysis, for the X and Y chromosomes. Our results with this method show correct sex prediction in 90% of male-bearing pregnancies (Oosterwijk et al. 1998*a*).

Multimode Microscopy

The concept of multimode microscopy is the combination of both bright-field and fluorescent microscopy. Bright-field microscopy is used for rapid screening of microscopic fields, for HbF-positive cells. This method of imaging is not photon limited, and images can be recorded at video rate (e.g., 25 images/s); therefore, the analysis time per slide is determined by the optical magnification (which determines the number of fields per slide) and by the scanning speed. Fluorescent imaging, which is generally slower than bright-field imaging, is used to record the nuclear counterstaining and/or the chromosome-probe signals of target cells.

Automated microscopy requires a system that consists of a microscope that is interfaced to a computer and equipped with a computer-controlled scanning stage, a motorized focus drive and objective rotor, and an automated filter wheel. In order to perform multimode microscopy, the microscope and its filters must be suitable for both bright-field and fluorescent microscopy. The computer-assisted microscope scans the slide, and, on the basis of preset characteristics (an algorithm), certain "events" are selected as potential NRBCs and stored in the computer's memory. The system must automatically correct its focus at a specified frequency. The selected objects are displayed on the computer screen and evaluated by the operator. In cases of doubt, the objects are again located automatically, by the click of the computer mouse, for further reevaluation by conventional microscopy.

Material and Methods

In the course of an ongoing study of the detection of fetal NRBCs in maternal blood (Oosterwijk et al. 1998*a*, 1998*b*), veinous blood samples (15 ml) were drawn, after informed consent was obtained, from pregnant women who had chorionic villi sampling (CVS) for advanced maternal age. Mean gestational age at sampling was 11 wks 3 d (range ±6 d). Twenty-six pregnant women participated in the study, of whom 16 were sampled both pre- and post-CVS. The study was approved by the hospitals' ethics committees.

The enrichment, preparation, and staining protocols have been described elsewhere (Oosterwijk et al. 1998*a*, 1998*b*). In short, a one-step enrichment was performed, within 5 h of sampling, by use of a triple Ficoll Hypaque (Sigma) gradient with densities of 1.077, 1.107, and 1.119 g/ml. Slide preparation was performed by centrifugal cytology. The cells were put onto glass slides by use of centrifugation buckets that contained ~1.5×10^6 cells. Centrifugation at 3,000 rpm (1,250 *g*) was performed in two steps, with BSA concentrations of 0.5% and 5% in the supernatants, respectively. The slides were spun dry with a Horizonter^R blood spinner (Hettich).

Staining for HbF was performed after fixation with methanol, acetone, and formaldehyde by a Cadenza automated immunostainer (Shandon). Slides were incubated with a monoclonal antibody for the Hb gamma chains (Immuno-rx). This was followed by incubation with a biotinylated goat antimouse secondary antibody (BioSPA) and streptavidin alkaline-phosphatase (AP) complex (BioSPA). The AP enzyme was developed with Vector-blue (Vector).

FISH on slides that contained NRBCs was carried out with a Y-chromosome–specific probe (satellite sequence III), directly labeled with Cy3, and the X-chromosome–specific probe pBamX5, directly labeled with flourescein isothiocyanate. FISH analysis was performed without prior knowledge of the CVS karyotype that served as a control. Figure 1 shows the results of immunocytochemical staining and FISH analysis on an NRBC detected in maternal blood sampled before CVS.

For manual screening, a conventional microscope (Leitz Laborlux) with a 20× objective was used. FISH manual evaluation was done on a Leica DM-RBE fluorescent microscope with a triple band–pass filter for simultaneous visualization of three fluorescent colors.

For automated image cytometry, a prototype system supplied by Applied Imaging Corporation was used. An extensive description of the system and the procedure is published elsewhere (Ravkin and Temov, in press). In brief, the system consists of a microscope with both transmission and fluorescent capabilities, a trinocular head, and 10×, 20×, and 40× objectives. In most instances, an Olympus BX-60 microscope (Olympus America) was used. A single-slide scanning stage (Maerzhauser Co.) is mounted on the microscope, with a 7-position transmission filter wheel, a 12-position fluorescence filter wheel, and a focus drive (TOFRA). All these

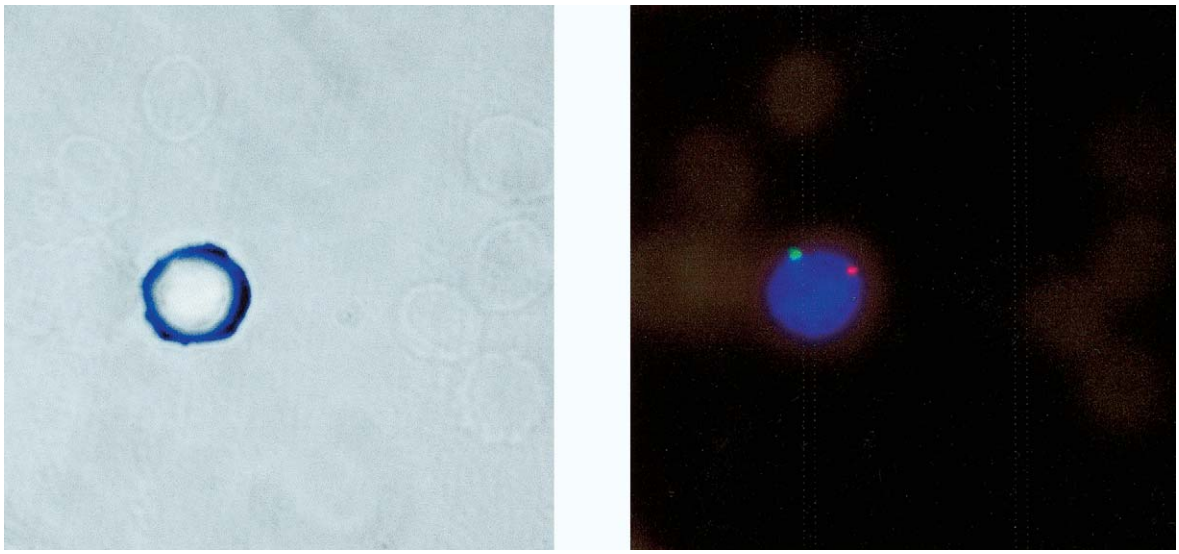

Figure 1 Digitalized microscopic image of fetal NRBCs detected in maternal blood, sampled before CVS. *Left*, HbF image in bright-field microscopy (Vector blue). *Right*, Same cell in fluorescence with nuclear staining (DAPI) and signals for the X and Y chromosomes.

devices are based on stepping motors and are controlled by microstepping motor controllers (Intelligent Motion Systems). Images are acquired by use of a video camera with light-integration capability (COHU 4910, Cohu, Inc.) and a custom-built frame-grabber board, which includes a 10-bit analog-to-digital converter and frame-averaging ability. A personal computer (Dell Poweredge sp590-2) is used to control all microscope functions, to perform image acquisition and processing, and to perform user-interface functions. Images were visualized on a Nanao T2.17 display. We specified that after every five fields the system would automatically correct its focus. For FISH analysis with X- and Y-specific probes, green, red, and blue 4,6-diamidino-2-phenylindol (DAPI) fluorescences were acquired sequentially with a Chroma 83000 triple band–pass beam splitter (Chroma Technology Corp.) and single-band excitation filters. The green, red, and blue signals were visualized simultaneously for visual observation.

In total, 44 slides were made, and all were screened both manually and automatically in a triple-blind fashion. Moreover, in a subset of slides that we examined, the number of NRBCs detected by automated image analysis was identical to that detected by manual screening. We also compared the yield of NRBCs in a subset of slides made with 20 × and 10 × objectives.

Results

A total of 44 slides were screened. Twenty-three slides were found to be positive (i.e., ⩾1 HbF-containing NRBCs) by automated screening, and 19 were found to be positive by manual screening. The results are summarized in tables 1 and 2. The number of fields screened by automated microscopy was within the range 3,128–5,876. The average number of objects selected by the system as possible fetal NRBCs was 1,452, with a range of 107–9,981 objects. The average number of actual fetal NRBCs, as determined by the operator when evaluating the gallery of selected objects, was 8 with a range of 0–111 cells. In the positive cases (⩾1 NRBC), the mean number of fetal NRBCs was 16.7 (range 1–111). The average time needed for screening was 4 h 27 min (range 1 h 20 min to 7 h 20 min). The average time needed for the operator to screen the automatically selected galleries was only 10 min (range 5–25 min). The average time needed for manual analysis of a slide was 20 min (range 10–45 min).

The ratio between the number of objects initially selected by the system and the number of actual fetal NRBCs from the gallery was 182:1 for the whole data set (44 slides), with a range of 9.4–∞ (no fetal NRBCs found in 3,000 objects). In the positive cases, the ratio of initially selected objects to fetal NRBCs from the gallery, confirmed by the operator, was 106:1 (range 9.4–6,071). This implies that, for these cases, 9–6,000 objects in galleries had to be screened by the operator in order to detect one fetal NRBC.

Of 44 slides analyzed by automated image analysis, 23 were positive (range 1–111 NRBCs; mean 16.7; median 3; mode 1). Of these 23 slides, 17 appeared to be positive by use of manual screening as well. By use of manual screening, 19 slides were positive (range 1–40 NRBCs; mean 11.3; median 2.5; mode 1). Two of these were found to be negative (no NRBCs) on the basis of automated image analysis. Twenty-five slides were found

Table 1

Results of Manual and Automated Analysis of 44 Slides, in Chronological Order

Slide	Manual Analysis: No. of NRBCs	Automated Analysis	
		No. of Objects	No. of NRBCs
419a	40	489	52
449b	1	6,071	1
449a	30	9,981	35
450b	0	187	0
451b	0	1,151	2
452b	0	300	0
452a	0	859	4
457b	0	402	0
457a	0	169	0
458b	0	840	0
459b	0	441	0
459a	12	1,997	22
485a	1	2,328	1
490a	0	1,996	0
495b	0	430	0
495a	0	243	2
496b	2	1,182	2
496a	1	991	3
502b	0	236	0
502b	0	147	0
503b	26	621	45
503a	40	2,319	111
510b	1	1,289	0
513b	0	121	0
513a	0	168	1
515b	0	553	0
521b	2	107	1
521a	2	460	4
531b	0	960	0
531a	12	385	10
532b	0	382	0
535b	0	614	0
535a	8	1,138	12
536b	1	2,987	0
536a	25	4,468	39
537b	0	508	0
537a	0	1,095	0
681b	0	385	0
681a	3	723	2
682b	1	134	1
691b	6	6,278	7
691b	0	3,210	3
691a	0	2,096	1
691a	0	3,436	0

Note.—a = sampled after CVS; b = sampled before CVS.

to be positive by use of either screening method or by use of both: in 16 cases (64%), automated analysis revealed more NRBCs than did manual screening; in 4 cases (16%), the results were identical; and in 5 cases (20%), manual screening produced a higher NRBC yield.

The comparison of screening results (i.e., the number of detected NRBCs) for the two methods, for the 25 positive slides, are summarized in figure 2. In the lower cell-count range ($\leq$15, where the cell count is defined as the mean of the manual and automated NRBC numbers), there was a very good correlation between results obtained from automated and manual screening. However, in the higher cell-count range (mean cell count >15), there was a discrepancy that increased, mainly in favor of automated image analysis. Regression analysis revealed that a curve was best fitted to the data as depicted in figure 2. The proportion of variation explained by the curve (Rsq) was 90%. The Wilcoxon signed-rank test, applied to the whole data set of 44 slides, demonstrated a significant difference in NRBC yield between automated and manual analysis, in favor of automated image analysis ($P = .0443$).

In eight randomly selected cases, we determined whether the NRBCs detected by automated image analysis and those detected by manual screening were indeed identical (i.e., whether the same cells were detected by either method). After automated image analysis was performed, the operator manually located the NRBCs and marked them by ink on the glass, in a way that did not interfere with the automated image analysis. The slides were then placed on the system again, and the NRBCs were relocated on the basis of the stored coordinates. These objects were checked for the presence of a circle or a dot. The data for these slides are shown in table 3. Automated analysis appeared to be more sensitive than manual analysis. The sensitivity of manual analysis was on average 55% (range 33–100), whereas the sensitivity of automated analysis was 91% (range 50–100), as depicted in the cross-table of detected cells (table 4). The discrepancies between manual and automated analysis increased as the average number of detected NRBCs increased.

FISH for the X and Y chromosomes was performed on 21 of the 23 slides that were found to be positive by automated image analysis. The FISH efficiency in control samples (NRBCs in male cord blood, stained and processed in identical fashion) was 85% and 70% for the X- and Y-chromosomal probes, respectively. The FISH data are shown in table 5. Of these 21 FISH results, two could not be compared with results derived from conventional karyotyping of the CVS: in one case, the woman had a miscarriage just before CVS, and, in the other case, there were no FISH signals detected because of technical problems. Of the remaining 19 cases, 13 (68%) showed that the predicted sex was concordant

Table 2

Performance of Manual and Automated NRBC Detection in the 44 Samples

Analysis	No. (%) of Slides, by No. of NRBCs Detected				
	0	1–4	5–9	10–24	⩾25
Manual	25 (57)	10 (23)	2 (5)	2 (5)	5 (11)
Automated	21 (48)	14 (32)	1 (2)	3 (7)	5 (11)

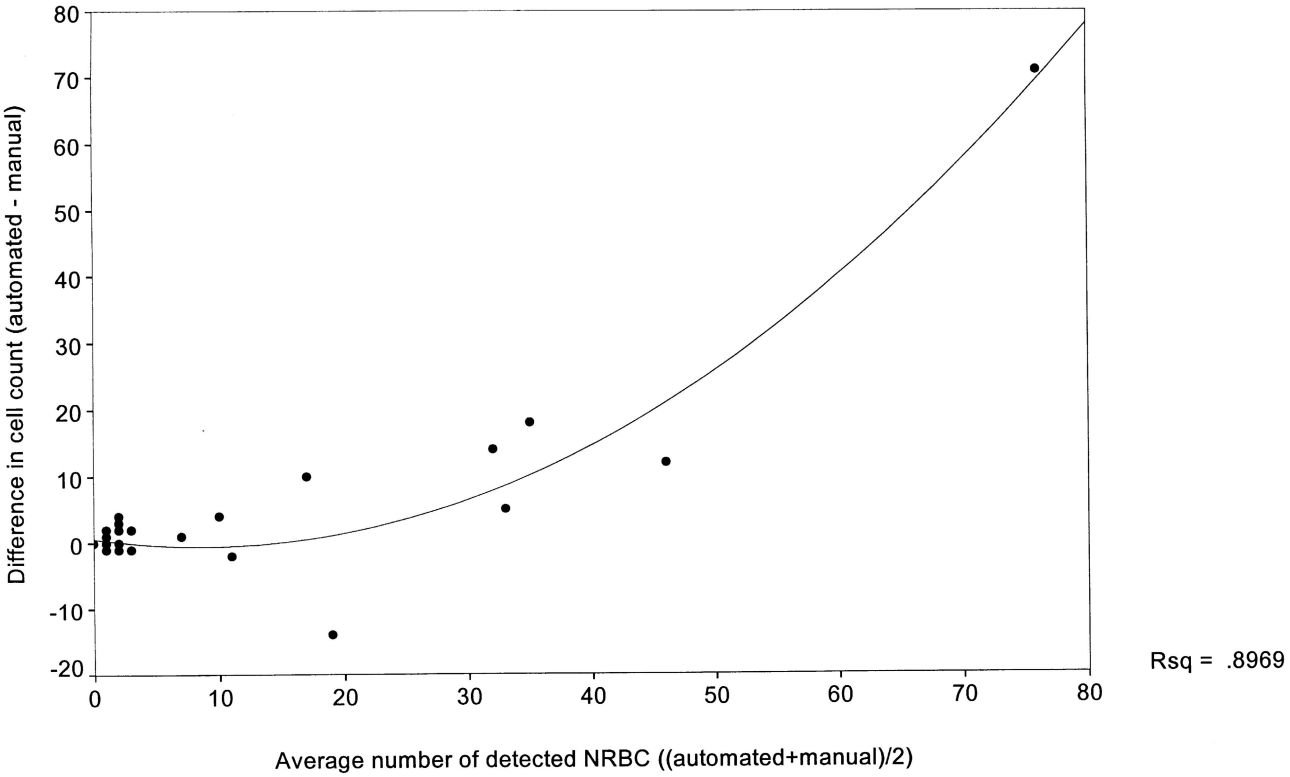

Figure 2 Correlation between manual and automated analysis of 25 slides with ⩾1 NRBC at either analysis or at both. Each slide is represented by one small dot; there are three overlapping dots in the very low cell-count range.

with the CVS results (i.e., the presence of XY signals in a male fetus and the presence of XX signals, without XY signals, in a female fetus). Of the male fetuses, 7 (64%) of 11 were concordant; of the female fetuses, 6 (75%) of 8 were concordant. In six cases, there was complete discordance between the FISH data and the CVS results. In three of these, the FISH result was 45,X; in two cases, FISH was XX when the fetus was male; and, in one case, FISH was XY when the fetus was female. In the discordant cases (6 [55%] of 11), the number of detected NRBCs was <8, whereas in all cases with ⩾10 NRBCs, the FISH data were concordant.

Discussion

The usual approach to the detection of fetal NRBCs in maternal blood is by means of enrichment, through either fluorescence-activated cell sorting or magnetic-activated cell sorting. In order to reduce cell loss and to preserve cell morphology, we adapted a protocol that was originally used for image cytometry after only ficoll density-gradient enrichment (Oosterwijk et al. 1998*b*). The protocol was tested on NRBCs obtained from CVS washings and seeded into lymphocyte suspensions, which showed that automated image analysis gave excellent cell-detection sensitivity. Cells were detectable at frequencies as low as 1 in 10^6 (Tanke et al. 1996). Moreover, the protocol appeared to be suitable for maternal blood samples obtained after CVS (Oosterwijk et al. 1998*a*). In this article, we have described a comparative study of manual and automated image analysis on maternal blood samples.

Automated microscopy performed significantly better than manual screening: the average NRBC count in positive cases was 16.7 (range 1–111) from automated analysis versus 11.3 (range 1–40) from manual screening. The percentage of positive cases was 43% for manual screening and 52% for automated image analysis. In the lower cell-count range (⩽15 NRBCs), which constituted 79% of positive cases, the results were practically identical. The good correlation between manual and automated cell detection in the low cell-count range may be a true phenomenon (i.e., there were no more NRBCs to detect), but it may also be statistically overestimated, because a maximum of two NRBCs per slide does not give much space for discordance. For this reason, the slides in which both methods failed to detect any NRBCs were excluded from figure 2. As depicted in table 1, the slides made of blood samples taken before CVS con-

tained on average very low numbers of NRBCs. In this low cell-count range, the advantage of the use of automated screening and image analysis will be mainly an increase in efficiency and not so much an increase in NRBC yield, since the sensitivities of automated and of manual NRBC detection are comparable.

In the higher cell-count range, however, automated image analysis was superior to manual screening. This might be because manual screening was performed with extreme alertness to detect the presence of any NRBCs at all, whereas for slides with many NRBCs alertness to detect all NRBCs may have flagged. The difference between automated and manual evaluation in the high cell-count range is relevant because (1) NRBCs should be detectable in a higher percentage of pregnant women and (2) there should be an increase in the number of detected NRBCs, which will improve the (statistical) reliability of the FISH diagnosis in each case.

The average number of objects detected by automated image analysis was 1,456, with a range of 100–10,000 objects. The thresholds for detection of target cells were deliberately set low so that cells would not be missed, with the calculated risk of detecting a relatively high number of false-positive cells. However, false-positive cells could easily be recognized as such by visual verification of the image memory. The wide variation in object number may indicate variabilities in specimen content (maternal blood-cell count), in specimen quality (freshness at processing), and in the basic physiology of hematologic parameters, all of which have to be addressed by a suitable algorithm. Further refinement of the preparation protocol may reduce this variability. The ratio between the number of objects selected by the system and the number of NRBCs selected by the operator when evaluating the gallery was on average 200:1 (range 9–∞) in all cases, and 117:1 (range 9–6,000) in positive cases. This may reflect variability in hematologic parameters as well as in background staining. Hence, both the absolute number of objects selected and the ratio between selected objects and selected NRBCs will eventually determine both the workload and the system efficiency.

When the outcomes of the two methods of NRBC detection are compared, the obvious question is whether the same NRBCs were detected by both. To answer this question, the NRBCs found by use of manual screening were marked in a random sample of eight slides and checked against the previously determined coordinates of the automated microscope. This comparison showed that automated image analysis was superior to manual screening with retrievals of 91% (range 50–100) and 55% (range 33–100), respectively. In the very low cell-count range, the overlap was nearly complete, but when the average NRBC number detected manually and automatically was ⩾4, manual analysis was less efficient. There may be several reasons to explain the detected discrepancies between manual and automated analysis. First, when many NRBCs are detected during manual screening, the technician may become less diligent and miss a fraction of the target cells. Second, when an

Table 4

Cross-Table of NRBCs Found by Manual Screening, Automated Screening, or Both, from Table 3

	No. of NRBCs Detected		
Result	Manual Positive	Manual Negative	Total
Automated positive	26	25	51 (91.1)
Automated negative	5	?	5 (8.9)
Total	31 (55.4)	25 (44.6)	56 (100)

Note.—The total number of detected cells in eight slides is 56. Twenty-six (46%) of these were found by both methods.

Table 3

Performance of Manual and Automated Slide Analysis on the Basis of Single-Cell Recognition

Slide	Manual[a]	Automated[b]	Overlap[c]	Missed Manual[d]	Missed Automated[e]	Total NRBCs[f]
1	6 (43)	12 (86)	4	8	2[g]	14
2	2 (50)	3 (75)	1	2	1[h]	4
3	13 (62)	21 (100)	13	8	…	21
4	3 (33)	8 (88)	2	6	1	9
5	2 (100)	2 (100)	2	…	…	2
6	2 (100)	2 (100)	2	…	…	2
7	1 (50)	2 (100)	1	1	…	2
8	2 (100)	1 (50)	1	…	1[h]	2

[a] No. of NRBCs found by manual screening (% of total no.).
[b] No. of NRBCs found by automated screening (% of total no.).
[c] No. of identical NRBCs found by both methods.
[d] No. of NRBCs not found by manual screening.
[e] No. of NRBCs not found by automated screening or by the technician.
[f] Total no. of NRBCs (manual + automated + overlap).
[g] Faint HbF signal.
[h] Clustered cells.

Table 5

Results of FISH Analysis for X- and Y-Chromosome Probes, on 21 Slides

Slide	No. of NRBCs		No of NRBCs, by FISH Result			No. of Lost Cells	No. of Cells without Signal	Sex (CVS)
	Manual Screening	Automated Screening	XX	XO	XY			
419a	40	52		6	41	4	1	XY
449b	1	1			0		1	XY
449a	30	35	1	10	4	17	3	XY
451b	0	2	2					NA
452a	0	4	2		2			XY
459a	12	22	13	9				XX
485b	1	1	1		0			XY
495a	0	2		2	0			XY
496b	2	2	0	2				XX
496a	1	3	2	1				XX
503b	26	45	1	18	26			XY
503a	40	111		64	47			XY
513b	0	1		1	0			XY
521b	2	1	0		1			XX
521a	2	4	3	1				XX
531a	12	10	9	1				XX
535a	8	12		3	9			XY
536a	25	39		25	12		2	XY
681a	3	2	2					XX
682b	1	1	1					XX
691b	6	7	4	1	0		2	XY

Note.—Underlined numbers represent the FISH column that corresponds with the result of the CVS karyotyping. NA = not available: because of missed abortion, no CVS was performed.

NRBC is detected manually, the cell is usually placed in the middle of the microscopic field, for inspection at a higher magnification. When the manual screening of the slide is continued thereafter, the coordinates of screening may have changed, which could cause parts of the slide to be missed. Third, technical imperfections such as overlapping cells or faint immunocytochemical staining may cause the algorithm to miss the object. Fourth, the operator may miss the NRBCs in the process of analyzing the selected objects in the gallery. However, intraobserver and interobserver variabilities appeared to be negligible in our data set and thereby excluded this factor as a possible cause.

FISH data on the detected NRBC population are insufficient to enable reliable statistical analysis. The observation that, in a number of XY pregnancies, NRBCs without Y signals were found may have two causes. First, a temporary problem with the Y probe that occurred during the study may explain four missed XY fetuses and could account for the high number of cells with only one X signal each in XY pregnancies (table 5). Second, maternal cell contamination may have given rise to NRBCs without Y signals. In the one case in which a Y signal was found in an XX pregnancy, this was most likely a result of nonspecific Y hybridization, although the theoretical possibility of residual Y-bearing NRBCs from previous pregnancies cannot be excluded. The general picture is that the predictive value of FISH analysis increases as the number of NRBCs detected increases: with $\geq$10 NRBCs, all XY fetuses were correctly diagnosed. Therefore, an increase in the number of detected NRBCs, the use of a more specific fetal NRBC staining, and any improvement of the FISH protocol all will facilitate reliable FISH diagnoses.

A fundamental parameter for the evaluation of automated image analysis is the time the system needs to analyze a slide. The average time needed for automated analysis ranged from 3 h 15 min to 7 h 20 min (mean 4 h 30 min; median 5 h), with use of a 20×-magnification objective. We screened the last seven slides of our data set by using a 10×-magnification objective. This did not lead to a reduction of selected objects, and the number of NRBCs per slide was similar; however, it did significantly reduce the screening time per slide (range 1 h 20 min to 2 h 20 min; mean 1 h 45 min; median 1 h 25 min). The mean of 1 h 45 min per slide plus 10 min for evaluation of the gallery by the technician is still much longer than what is needed for manual screening, which took a fully committed and experienced operator on average 20 min (range 10–45 min). It should be noted that the software used was primarily developed for testing the performance of the system and has not yet been

optimized for speed. Further development of dedicated software and hardware will reduce screening time per slide and also will improve the detected object/NRBC ratio, thereby reducing the time needed for verification of the galleries. Moreover, automated microscopy and the use of slide feeders enables slide screening for 24 h/d, 7 d/wk, which will greatly improve screening efficiency. Most important, when more specific markers for fetal NRBCs become available, the algorithm can be adapted, and the performance of automated analysis will also improve substantially.

To conclude, we have demonstrated that automated image analysis is applicable to detection of Hb-expressing NRBCs in maternal blood after minimal enrichment. It reduces the screening workload for the technicians and is more sensitive than manual evaluation, especially in the higher cell-count range: more slides are positive for NRBCs at a higher average number of NRBCs per slide. This enhances NRBC yield, improves FISH diagnoses, and will facilitate future clinical application of fetal cell diagnosis. Given the ongoing reduction in time needed for automated screening, we feel that automated image analysis will be helpful in the avoidance of complicated enrichment procedures.

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References

Bhat NM, Bieber MM, Teng NNH (1993) One-step enrichment of nucleated red blood cells: a potential application in perinatal diagnosis. J Immun Meth 158:277–280

Bianchi DW (1995) Prenatal diagnosis by analysis of fetal cells in maternal blood. J Pediatr 127:847–856

——— (1998) Fetal DNA in maternal plasma: the plot thickens and the placental barrier thins. Am J Hum Genet 62: 763–764

Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA (1990) Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci USA 87:3279–3283

Bianchi DW, Klinger KW, Vadnais TJ, DeMaria MA, Shuber AP, Skoletsky J, Midura P, et al (1996) Development of a model system to compare cell-separation methods for the isolation of fetal cells from maternal blood. Prenat Diagn 16:289–298

Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP (1997) PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. Am J Hum Genet 61:822–829

Bjorkqvist AM, Wessman M, Vuorinen M, Ylinen K, Keinanen M, Slunga Tallberg A, Knuutila S (1994) ISH and PCR study with Y-specific probe/primers. Ann NY Acad Sci 731:57–66

Cheung M-C, Goldberg JD, Kan YW (1996) Prenatal diagnosis of sickle cell anemia and thalassemia by analysis of fetal cells in maternal blood. Nat Genet 14:264–268

Cupp JE, Leary JF, Wood JCS, Doherty RA (1984) Rare-event analysis methods for detection of fetal red blood cells in maternal blood. Cytometry 5:138–144

Ganshirt D, Garritsen HS, Holzgreve W (1995) Fetal cells in maternal blood. Curr Opin Obstet Gynecol 7:103–108

Ganshirt-Ahlert D, Burschyk M, Garritsen HS, Helmer L, Miny P, Horst J, Schneider HP, et al (1992) Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 166:1350–1355

Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H (1993) Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet 91:427–432

Herzenberg LA, Bianchi DW, Schroder J, Cann HM, Iverson GM (1979) Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. Proc Natl Acad Sci USA 76:1453–1455

Langlois S, Wilson RD (1993) Noninvasive prenatal fetal testing by analysis of maternal blood. Clin Invest Med 16: 333–338

Leif RC (1983) Centrifugal cytology buckets. Cytometry 3: 311

Lewis DE, Schober W, Murrell S, Nguyen D, Scott J, Boinoff J, Simpson JL, et al (1996) Rare-event selection of fetal nucleated erythrocytes in maternal blood by flow cytometry. Cytometry 23:218–227

Liou JD, Pao CC, Hor JJ, Kao SM (1993) Fetal cells in the maternal circulation during first trimester in pregnancies. Hum Genet 92:309–311

Lloyd-Evans P, Kumpel BM, Bromelow I, Austin E, Taylor E (1996) Use of a directly conjugated monoclonal anti-D (BRAD-3) for quantification of fetomaternal hemorrhage by flow cytometry. Transfusion 36:432–437

Lo YMD, Bowell PJ, Selinger M, Mackenzie IZ, Chamberlain P, Gillmer MD, Elliott P, et al (1994) Prenatal determination of fetal rhesus D status by DNA amplification of peripheral blood of rhesus-negative mothers. Ann NY Acad Sci 731: 229–236

Lo YMD, Corbetta N, Chamberlain P, Rai V, Sargent IL, Redman CWG, Wainscoat JS (1997) Presence of fetal DNA in maternal plasma and serum. Lancet 350:485–487

Lo YMD, Patel P, Baigent CN, Gillmer MD, Chamberlain P, Travi M, Sampietro M, et al (1993) Prenatal sex determination from maternal peripheral blood using the polymerase chain reaction. Hum Genet 90:483–488

Lo YMD, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, Wainscoat JS, et al (1998) Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 62: 768–775

Mansi JL, Mesker WE, McDonnel T, Driel-Kulker AMJ, Ploem JS, Coombes RC (1988) Automated screening of bone mar-

row smears for micrometastasis. J Immunol Methods 112: 105–111

Mesker WE, van der Burg MJM, Oud PS, Knepfle CFHM, Ouwerkerk–van Velzen MCM, Schipppers NW, Tanke HJ (1994) Detection of immunocytochemically stained rare events using image analysis. Cytometry 17:209–215

Oosterwijk JC, Mesker WE, Ouwerkerk–van Velzen MCM, Knepfle CFHM, Wiesmeijer CC, Beverstock GC, van Ommen GJB, et al (1998*a*) Fetal cell detection in maternal blood: a study in 236 samples using erythroblast morphology, DAB and HbF staining, and FISH analysis. Cytometry 32:178–185

Oosterwijk JC, Mesker WE, Ouwerkerk–van Velzen MCM, Knepfle CFHM, Wiesmeijer CC, van der Burg MJM, Beverstock GC, et al (1998*b*) Development of a preparation and staining method for fetal erythroblasts in maternal blood: simultaneous immunocytochemical staining and FISH analysis. Cytometry 32:170–177

Ploem JS, Van Driel-Kulker AMJ, Goyarts-Veldstra L, Ploem-Zaaijer JJ, Verwoerd NP, van der Zwan M (1986) Image analysis combined with quantitative cytochemistry. Histochemistry 84:549–555

Ploem-Zaaijer JJ, Mesker WE, Boland GJ, Sloos W, van de Rijke FM, Jiwa M, Raap AK(1994) Automated image cytometry for detection of rare antigen–positive cells in peripheral blood. Cytometry 15:199–206

Ravkin I, Temov V. Automated microscopy system for detection and genetic characterization of fetal nucleated red blood cells on slides. In: Proceedings SPIE: advanced techniques in analytic cytology (in press)

Saunders AM, Zarowitz MA, Harrell P (1997) Fetal cells from maternal blood: design of a product. Lab Hematol 3: 282–291

Simpson JL, Elias S (1994) Fetal cells in maternal blood: overview and historical perspective. Ann NY Acad Sci 731:1–8

Simpson JL, Lewis DE, Bischoff FZ, Elias S (1995) Isolating fetal nucleated red blood cells from maternal blood: the Baylor experience. Prenat Diagn 15:907–912

Sohda S, Arinami T, Hamada H, Nakauchi H, Hamaguchi H, Kubo T (1997) The proportion of fetal nucleated red blood cells in maternal blood: estimation by FACS analysis. Prenat Diagn 17:743–752

Suzumori K, Adachi R, Okada S, Narukawa T, Yagami Y, Sonta S (1992) Fetal cells in the maternal circulation: detection of Y sequence by gene amplification. Obstet Gynecol 80:150–154

Tanke HJ, Oosterwijk JC, Mesker WE, Ouwerkerk–van Velzen MC, Knepfle CF, Wiesmeijer CC, van Ommen GJ, et al (1996) Detection of "rare event" fetal erythroblasts in maternal blood using automated microscopy. Early Hum Dev Suppl 47:S89–S93

Thomas MR, Tutschek B, Frost A, Rodeck CH, Yazdani N, Craft I, Williamson R (1995) The time of appearance and disappearance of fetal DNA from the maternal circulation. Prenat Diagn 15:641–646

van Driel-Kulker AM, Ploem-Zaaijer JJ, van der Zwan–van der Zwan M, Tanke HJ (1980) A preparation technique for exfoliated and aspirated cells allowing different staining procedures. Anal Quant Cytol 4:243–246

Verwoerd NP, Bernini LF, Bonnet J, Tanke HJ, Natarajan AT, Tates AD, Sobels FH, et al (1987) Somatic cell mutations in humans detected by image analysis of immunofluorescently stained erythrocytes. In: Burger G, Ploem JS, Goerttler K (eds) Clinical cytometry and histometry. Academic Press, San Diego, pp 465–469

Zheng YL, Carter NP, Price CM, Colman SM, Milton PJ, Hackett GA, Greaves MF, et al (1993) Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. J Med Genet 30:1051–1056