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Review

Current applications of single-cell PCR

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Abstract. The advent of the polymerase chain reaction (PCR) has revolutionised the way in which molecular biologists view their task at hand, for it is now possible to amplify and examine minute quantities of rare genetic material: the limit of this exploration being the single cell. It is especially in the field of prenatal diagnostics that this ability has been readily seized upon, as it has opened up the prospect of preimplantation genetic analysis and the use of fetal cells enriched from the blood of pregnant women for the assessment of single-gene Mendelian disorders. However, apart from diagnostic applications, single-cell PCR has proven to be of enormous use to basic scientists, addressing diverse immunological, neurological and developmental questions, where both the genome but also messenger RNA expression patterns were examined. Furthermore, recent advances, such as optimised whole genome amplification (WGA) procedures, single-cell complementary DNA arrays and perhaps even single-cell comparative genomic hybridisation will ensure that the genetic analysis of single cells will become common practice, thereby opening up new possibilities for diagnosis and research.

Key words. PCR; single cells; diagnosis.

Diagnostic applications

Preimplantation genetic diagnosis

The area of diagnostic science most affected by the advent of single-cell polymerase chain reaction (PCR) has undoubtedly been preimplantation genetic diagnosis (PGD), which was first used to determine the sex of single cells biopsied from human preimplantation embryos for X-linked genetic disorders [1]. This pioneering work was rapidly expanded to investigate numerous other single-gene disorders, including cystic fibrosis, the haemoglobinopathies and Duchenne's muscular dystrophy, and is now performed in several countries [2, 11]. This practice is, however, burdened by considerable social concerns, and consequently it is prohibited in several countries, including Germany and Switzerland [3, 4]. Nonetheless, it should, be borne in mind that the fear that the introduction of PGD would lead to a new wave of eugenics is largely unfounded, since those couples seeking such assistance usually have a very high risk of bearing a fetus with a genetic malfunction. Furthermore, since PGD is a very complex technique and can only be performed on preimplantation embryos obtained by in vitro fertilisation, it is unlikely that it will become a widespread procedure. Indeed, by the end of 1996 only about 600 instances of PGD had been reported worldwide [5]. Although most of these examinations were performed to screen for X-linked disorders such as Duchenne's muscular dystrophy, fragile X syndrome or haemophilia A, other inherited conditions that were examined include autosomal dominant disorders such as Marfan's syndrome and Huntington's chorea or autosomal recessive disorders such as cystic

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fibrosis, Tay-Sachs disease and the haemoglobinopathies [5].

In general, most PGD protocols involve the removal of one or two cells from the 6–10 cell embryo, which is generally on the 3rd day after oocyte pickup [6–8]. Prior to biopsy, care has to be taken to flush the embryo free of all maternal (cumulus cells) and paternal (sperm) contaminants. The removal of single embryonal cells is achieved by grasping the embryo with a fine holding pipette and then puncturing a small hole into the zona pelucida either with a sharp needle, or acid hydrolysis and immediate neutralisation, or by laser dissection and subsequent aspiration of the required numbers of cells with a micropipette [7].

These single embryonal cells can then either be analysed by FISH (fluorescence in situ hybridisation) for the most common chromosome aneuploidies (21, 18, 13, X and Y), for which commercial kits are now available that permit the simultaneous analysis of all five chromosomes or by PCR for the gene locus in question [9, 10].

Since only one or two cells are available for analysis, strategies have been developed to maximise the amount of information retrieved and to ensure that the highest degree of accuracy is attained [11–15]. As with most other applications dealing with the analysis of single cells, these have focused on the use of multiplex PCR reactions, whereby using of different sets of PCR, primers multiple loci can be simultaneously interrogated [16–18]. In this manner, methods have been established which permit the simultaneous analysis of four or more loci, such as short tandem repeats (STRs), the amelogenin locus on the X and Y chromosomes for fetal sexing and the genetic disease locus in question. A technical advance which has been of great assistance here, and which is discussed in more detail below, is that of fluorescently labelled PCR, since this allows single-base-pair size discrimination of PCR products [16, 19].

Even though one can be reasonably sure that one is dealing with pure fetal material, the use of STRs, also termed microsatellites, is strongly advocated to ensure that no contamination of the sample has taken place and, perhaps most important, to ensure that the cell analysed is indeed taken from the embryo in question. For this reason several labs will additionally genotype the parents as well, to ensure that they are dealing with one of their offspring.

As with most diagnostic assays, it has also been the experience in this field, that assays should be developed in such a manner that a misdiagnosis does not result from lack of PCR amplification, as happened in several instances where fetal sexing was performed on preimplantation embryos [5]. Thus, adequate controls should always be included to ensure that the PCR reaction was functional, for instance by coamplifying X-chromosome-specific sequences, when performing assays to determine fetal sexing.

To counter the effect of allele dropout (ADO), whereby only one allele is underrepresented due to inefficient amplification, it is best to examine two cells from the same embryo, and only transfer those where a concordant result is obtained on both cells examined [11, 16, 20, 21]. If a gene deletion is analysed, the assay has to be designed in such a manner that in cases with the deletion, no PCR product is present. In this way no affected embryo will be accidentally transferred [11].

In order to retrieve the maximum amount of information from these one or two fetal cells, a further technique, also termed cell recycling, has been devised whereby cells are sequentially analysed by PCR and then by FISH [22]. A problem with this method is that it appears to very prone to ADO [23].

Fetal cells isolated from maternal blood

Enrichment and isolation

Prenatal diagnosis of fetal chromosomal aberrations or genetic disorders currently involves invasive procedures such as amniocentesis or chorionic villus sampling. Due to the invasive nature of these practices and their procedure-associated risk, they are only offered to high-risk populations. Hence, a considerable need exists for a safe but efficacious noninvasive alternative [24]. Since during pregnancy fetal haemopoietic cells enter the maternal bloodstream, one way of achieving this goal is by the enrichment and isolation of such fetal cells. As these fetal cells are rare, being present at a frequency of less than 1 in a million maternal cells, their enrichment represents a considerable challenge and is usually accomplished either by FACS (fluorescent-activated cell sorting) [25] or the magnetic alternative MACS (magnetic cell sorting) [26]. Recently, however, various other approaches have been tested, including minimal enrichment step Percoll gradients [27, 28] or charge flow separation [29].

The cell type favoured by most researchers is the erythroblast, as it can be readily identified by its characteristic morphology of a dense nucleus, clear cytoplasm and size of a mature erythrocyte [10]. Furthermore, by the high-level expression of several antigens, which include the transferrin receptor (CD71), the blood group antigen glycophorin A (GPA) or the fetal and embryonic haemoglobins, the enrichment of this cell type is more readily facilitated [30, 31]. The latter can also be used for the preliminary identification of fetal erythroblasts [10, 32, 33].

For the analysis of single enriched fetal erythroblasts, most researchers have opted for the same types of

techniques as described above for PGD, using either one or two micromanipulators. In the first published report on the analysis of single fetal cells Takabayashi and colleagues described a method [27] by which the fetal erythroblast was first gently lifted from the microscope slide with a finely pulled needle and then subsequently drawn into a transfer pipette by suction. The single cells were then expelled into a PCR reaction vessel by the application of positive pressure. This procedure was successfully used to isolate single fetal cells for the analysis of rhesus D and Duchenne's muscular dystropy [34, 35]. For the analysis of haemoglobinopathies, on the other hand, Cheung in the laboratory of Y. W. Kan used a single microneedle to lift individual fetal cells from the slide [36]. In both these procedures, stained and fixed cells were allowed to gently loosen from the slide by the application of a drop of sterile water or phosphate-buffered saline (PBS). Von Eggelling and colleagues, on the other hand, applied the enriched cells in a droplet of buffer onto a glass slide, and then using a microcapillary carefully aspirated the cell with the desired morphology using phase contrast microscopy [37].

In our laboratory, we have tried several approaches, including the scraping of single stained cells from the glass slide with a single glass needle [16, 21], to a two-device micromanipulation system, before finally settling on an approach whereby the cells are gently loosened by a drop of PBS and then are individually skewered onto a finely drawn glass needle (fig. 1) [31]. Following successful cell pickup, which can be monitored under the microscope, the needle is broken off directly into a PCR reaction vessel to ensure that successful transfer of the cell has taken place. More recently, we have investigated the use of a laser-mediated micromanipulation system, which is discussed in more detail below [X. Y. Zhong et al., in preparation].

Microsatellites and the genetic identification of fetal cells

Even though the fetal cell of choice, the erythroblast, is rare in the normal adult periphery and can potentially be identified by the use of fetal specific antigens such as the fetal or embryonic haemoglobins, one needs to be absolutely certain that the cell analysed is indeed fetal.

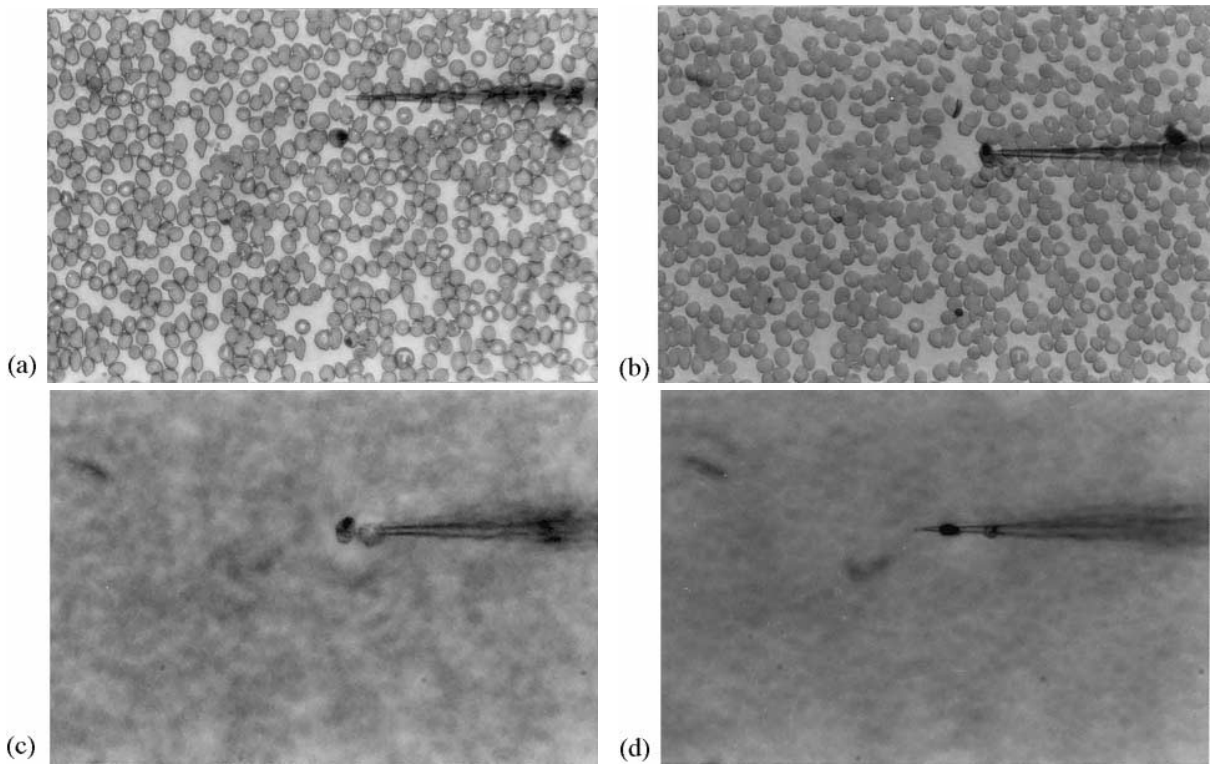

Figure 1. Micromanipulation of a single erythroblast using a finely drawn glass capillary. This figure illustrates the actual colocalisation of the microcapillary and the desired single cell (*a*), the piercing of the single erythroblast with the fine needle (*b*) and the actual lifting of the single erythroblast off the microscope slide (*c*) with it attached to the tip of the needle (*d*).

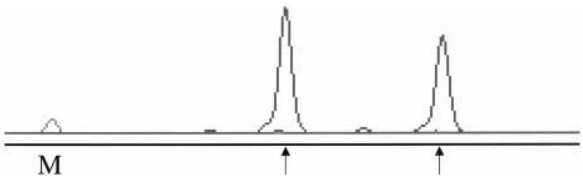

Figure 2. Fluorescent quantitative SC-PCR analysis of microsatellite loci. This analysis shows that both microsatellite loci (indicated by arrows) can be detected following SC-PCR analyses, but that the expected 1:1 quantitative ratio of the area under the two peaks is no longer retained. Hence, this technique cannot be reliably used on single cells for the determination of chromosomal ploidy. The molecular weight marker is indicated by M.

This is especially relevant in the field of prenatal diagnosis, which can involve decisions regarding termination of pregnancy. The only way we felt that this could be irrefutably demonstrated is by a genetic analysis using highly heterozygous loci, such as STRs (short tandem repeats).

For this purpose we have developed a multiplex fluorescent PCR reaction wherein at least four to six such STR loci can be simultaneously analysed [16]. In our experience it is best to first genotype both the mother and father in order to determine which loci are informative: these loci are then used to determine whether the cell being examined is of fetal or maternal origin. The reason for typing both parents is to be certain that the fragment sizes determined in the analysed single cell do indeed correspond to the size pattern of both parents, and are not the result of some PCR artefact, which occurs quite frequently for these highly polymorphic loci. This is probably caused by Taq polymerase stuttering due to the high number of short repeat sequences these loci contain.

Using this approach we were readily able to prove that cells were fetal in a model system in which cord blood had been diluted into maternal blood [16]. In a similar approach using a cord blood model system, the group of Ferguson Smith has been able to determine fetal sex, microsatellite loci and mutations in the cystic fibrosis gene [38].

On the other hand, von Eggeling and colleagues used such highly polymorphic markers for the identification of fetal cells which had been enriched from blood samples taken from pregnant women [37].

A further advantage of the use of STRs is that since each locus is representative of a particular chromosome, this analysis can give an indication of the ploidy for the chromosome being examined (fig. 2). In this way, if two separate maternal chromosomes are inherited in addition to the paternal one, which is the instance in meiosis I errors, three different PCR products will be obtained. If, however, the same maternal chromosome is inherited in duplicate, which occurs in about 23% of all cases [39, 40], then only a PCR product for this maternal chromosome and a separate one for the paternal one will be obtained. Quantification of the relative levels of each PCR product is only possible in a reliable manner when there is ample template at the beginning of the PCR reaction. Under these conditions a 2:1 ratio would be shown to exist between, thereby indicating the presence of two copies of the maternal allele. In this manner fluorescent quantitative PCR can be used to distinguish between di-allelic and tri-allelic trisomies. This has been successfully applied for the analysis of chromosomal ploidy using PCR specific for STR loci on chromosome 21 on both chorionic villi and amniocytes [41, 42]. These conditions, however, do not apply to the analysis of single cells, as here there are only two to three copies of the template which are associated with the problem of ADO or preferential amplification [21, 43]. As such, STRs can only be used as being indicative of fetal chromosome ploidy when the fetus is tri-allelic, since under these conditions due to the phenomenon to preferential amplification, an observation also made by Sherlock et al. [43] (fig. 2).

Successful prenatal diagnosis using fetal cells from maternal blood

As alluded to above, fetal cells isolated from maternal blood have been used for the diagnosis of several genetic loci, including rhesus D, Duchenne's muscular dystrophy, ornithine transcarbamylase deficiency, spinal muscular atrophy and globin genes [34–36, 44, 45]. However, several of these studies are not strictly speaking single-cell analysis, since although single cells were micromanipulated, several authors have pooled these cells for the final PCR analysis in an attempt to overcome the problem of ADO [36]. The major concern of this approach is that a high risk exists of contaminating the final preparation with an erroneously picked maternal erythroblast.

In the studies published by Sekizawa and colleagues [34, 35], single cells were examined for both fetal sex, rhesus D and for mutations causing Duchenne's muscular dystrophy. In these studies, single and not multiplex PCR reactions were performed. To permit the analysis of multiple loci, these authors used the primer extension preamplification (PEP) technique, by which the genome is randomly amplified [46, 47]. Such whole genome amplification (WGA) procedures can yield sufficient template for up to 50 different further analyses. They should, however, be used with some caution, as they may lead to unequal amplification of all alleles. Indeed, we have observed ADO to occur during PEP treatment [21].

In order to test the efficacy of isolated fetal cells for prenatal diagnostic purposes, we have turned to a simple model system, namely a multiplex PCR analysis for fetal sex, using the SRY locus and rhesus D status [48]. Since these loci are absent in the genome of rhesus D pregnant women, this system would allow rapid screening of numerous single erythroblasts. To ensure that genetic material was indeed present and that the PCR reaction was functional, we also amplified a region from the β-globin gene, which is present in all genomes (fig. 3).

Furthermore, since during pregnancy both maternal and fetal erythroblasts are present in the circulation of the pregnant women, an additional question we wished to address with this system was which fraction of erythroblasts in this instance was of fetal origin.

In our study in which we recruited 19 patients, erythroblasts could only be enriched in 14 of these, even though we were using a protocol that we had recently established to have a very high efficiency. From these 14 cases, on average 9 single erythroblasts were micromanipulated and analysed by the single-cell multiplex PCR reaction. In all instances, we were able to correctly determine the fetal genotype for both fetal sex and rhesus D status. This study currently represents the most extensive characterisation of fetal loci by noninvasive means [48].

Use of single-cell PCR for nondiagnostic applications

Interest in examining the genetic nature of single cells has not been restricted to the field of prenatal diagnostics, but has also been seized upon by researchers addressing developmental or clinically oriented research.

In order to genetically distinguish two different cells from the same individual, these cells, which are in essence genetically identical, have to differ at some point. For such a genetic disparity to occur, some form of gene rearrangement would have to take place. Examples of such instances are the B and T cell lymphocyte receptors (BCR and TCR, respectively) [49], loss or gain of heterozygosity in tumour cells [50] and virus-infected cells [51]. Consequently, single-cell PCR has made the largest impact in the fields of immunology and oncology, where it has been used to examine changes in the B- or T-cell-receptor repertoire or to determine the clonal origin of tumours.

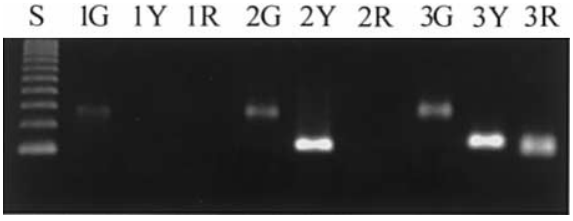

Figure 3. Simultaneous analysis of the rhesus D, SRY and β-globin loci on single cells. PCR products are indicated by the following key: S, 100-bp DNA molecular weight marker; G, β-globin PCR product; Y, SRY gene PCR product; R, rhesus D gene PCR product. The first cell is, hence, maternal, as no amplification occurred for the rhesus D and SRY loci. The second cell is from a female fetus as it is positive for only two loci, whereas the third cell is from a male rhesus D positive fetus as it is positive at all three loci.

During B and T lymphocyte development their respective receptors are generated by the splicing together of the required variable, joining and constant domains, thereby yielding a unique receptor or immunoglobulin molecule [49]. Tumours, on the other hand, arise by the accumulation of multiple genetic hits or lesions, which can involve chromosomal rearrangements or point mutations and which either lead to the activation of oncogenes or loss of tumour suppressor genes [50]. These genetic alterations permit the necessary degree of discrimination between normal, premalignant and tumour cells.

In this manner ten Boekel and colleagues [52] established that the expression of the $\lambda 5$ surrogate immunoglobulin (Ig) light chain is required to establish allelic exclusion of the IgH, and that the expression of the pre-BCR is important in determining the IgH repertoire.

In an examination of the role of the pre-TCR in determining the fate of β chain rearrangement, Aifantis and colleagues [53] observed an ordered system of rearrangement of the β chain akin to that of the IgH chain in B cells, in that only one allele is subject to rearrangement. If this does not form a functional pre-TCR with its α chain partner, then rearrangement of the second β chain is initiated.

Single-cell PCR has furthermore been used to examine the TCR repertoire of CD8+ T cells in response to antigenic challenge [54]. Here the overall repertoire was estimated to be in the range of 15–20 different clones. It is noteworthy that the same repertoire was displayed by the memory T cells as examined following a subsequent antigenic challenge.

Since tumours arise from a series of genetic events which uncouple a cell's response to normal growth control, these have also been addressed at a single-cell level. These analyses were performed to determine the degree of clonality within a given tumour, but also the origin of malignancy in patients suffering a relapse.

In this manner SC-PCR examinations of Hodgkin and Reed-Sternberg (H-RS) cells, the putative malignant cells in Hodgkin's disease, have not only shown that these cells regularly bear the characteristic clonal immunoglobulin rearrangements, but that these cells also

persist despite harsh treatment regimens and are present in patients with a relapse [55].

In the instance of adult T cell leukemia (ATL), SC-PCR has been used to address the question of abnormal T cell morphology present in this disease and the extent of human T lymphotropic virus type l (HTLV-l) infection. In the study performed by Miyagi and colleagues [51], in which both single morphologically normal cells and those with an indented and lobulated nucleus were examined by SC-PCR for the presence of integrated HTLV-l sequences, it was shown that these viral sequences were present in both lymphocyte groups, thereby indicating that the extent of viral infection was much greater than previously perceived to be.

Single-cell RT-PCR

The ability to analyse gene transcription at a single-cell level has permitted developmental biologists to examine expression patterns in single cells during differentiation. In this manner, using a multiplex single-cell RT-PCR assay which permitted the analysis of six different genes simultaneously, Cornelison and Wold [56] examined changes in expression of the MyoD family during the transition of skeletal muscle satellite cells following stimulation and subsequent differentiation.

Single cell RT-PCR has, however, also helped to shed new light on the regulation of gene expression, for it was by these means that Holländer and colleagues [57] could demonstrate mono-allelic expression of the murine interleukin (IL)-2 gene. This was achieved by exploiting a polymorphic difference between the IL-2 gene of *Mus musculus* and *Mus spretus*, and by examining single T cells obtained from such hybrid mice, which showed that these cells either express the *M. spretus* allele or the *M. musculus* allele. Since the IL-2 gene is located on an autosomal nonimprinted locus, this unusual result gives a new insight into the complex mechanism regulating critical genes.

In a technically more demanding approach Malnic and colleagues [58] used a combination of SC-RT-PCR and calcium imaging to examine the expression of particular odour receptors, which gave an indication of the complexity with which particular odors are perceived, in that they found that although one odorant can be recognised by multiple odor receptors, these receptors are also capable of recognising different structurally related odors. This implies that the olfactory system types the identity of a particular odor in a combinatorial manner.

A publication that pushed the limits of current technology and thus gives an indication of where the field is heading is the report by Fink and colleagues [59], who quantitated TNF-α mRNA accumulation in alveolar macrophages. Single cells were obtained by laser-assisted micromanipulation from broncheolar lavage specimens. In these cells the number of TNF-α transcripts was determined by the use of Taqman realtime quantitative PCR technology [60]. In addition, using a different laser system, Bernsen and colleagues [61], as well as Fend and colleagues [62], were recently able to detect mRNA expression in immunostained cryosections.

Although these studies are strongly suggestive of future trends, they do suffer from the same drawback that most researchers have experienced: that RT-PCR is best performed on freshly isolated single cells, and that if fixed or stained preparations are used, then pools of cells have to be used.

In our experience, cells fixed with either ethanol or methanol tend to give superior results than those fixed with acetone or formaldehyde-based fixatives. These are, however, rather empirical observations, and those planning to use an SC-RT-PCR approach should best determine the optimal conditions for their specified cell type.

Laser-mediated micromanipulation systems

As briefly alluded to above, novel laser forms of microdissection are making possible the isolation of single cells from complex multicellular tissue sections. The main distinction between two of the laser-assisted cell-picking systems currently promoted is the manner in which the desired cells are isolated.

In the system developed by Liotta and colleagues [63, 64], termed laser capture microdissection, the pulse of laser energy physically attaches the cell on which the laser was focused onto a synthetic membrane suspended directly above the cell preparation. Since this membrane is melted by the laser pulse, the resulting stickiness covalently bonds the cell to the membrane. Upon removal of the membrane, which is conveniently applied to the cap of a special PCR reaction vessel, only those cells which have been bonded to the membrane are transferred. Since it seems difficult to finely focus the laser to the size and shape of a single cell, it is in our opinion not really feasible to obtain a pure single-cell preparation using this system.

The other system, pioneered by Schütze and colleagues [65, 66], relies on the presence of a pulsed 337-nm ultraviolet laser. Here the cells are first transferred onto a microscope slide covered by a thin polyethylene membrane. Individual cells are localized under the microscope and excised by circumcision with a high-energy focused laser beam. The extremely high photon density within the laser focus can be used to cut or to ablate biological structures. The desired isolated cell is ejected from the substrate with a single precisely pointed laser shot and lifted directly into the cup of a common PCR

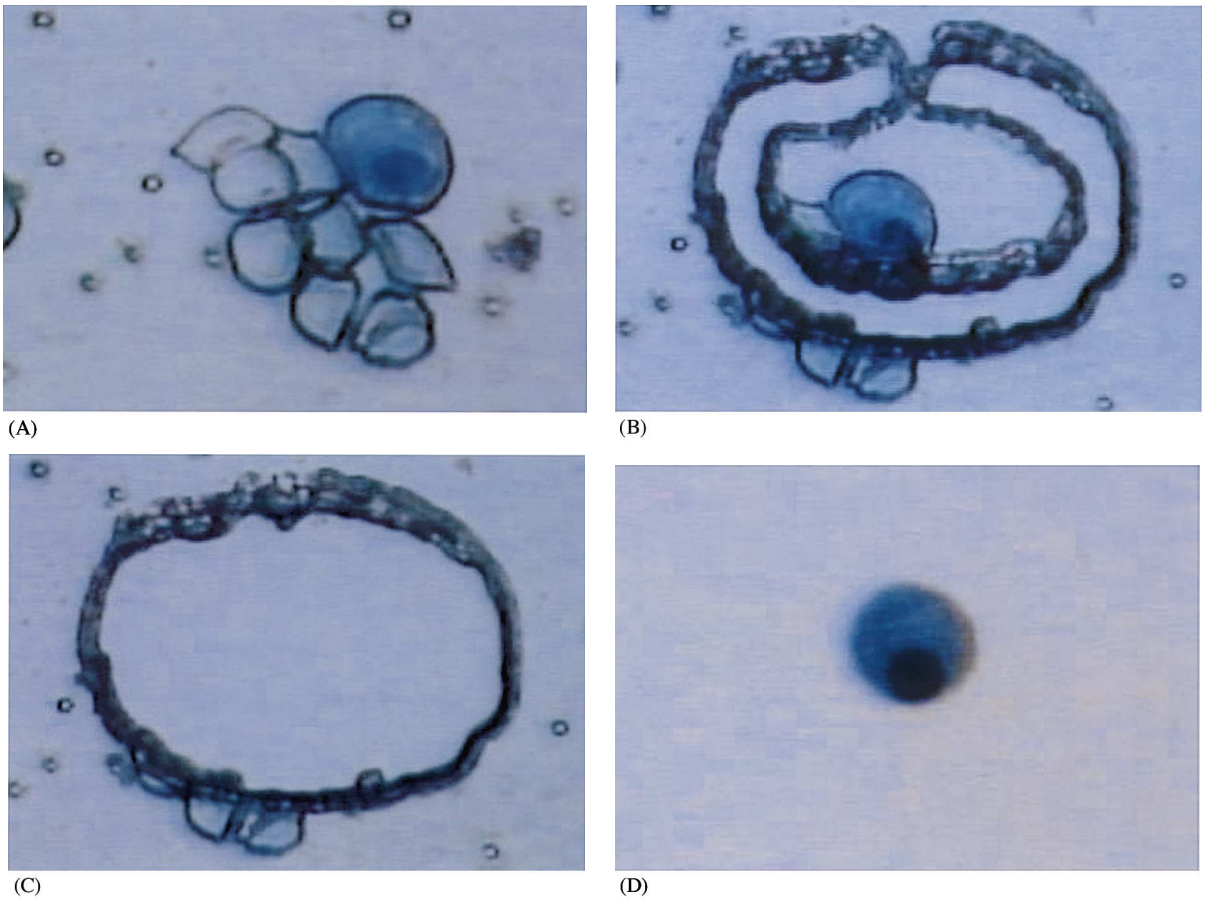

Figure 4. Laser-mediated micromanipulation. This figure illustrates localisation of the desired erythroblast in a cluster of potentially contaminating erythrocytes (*a*), the laser-mediated circumcision of this cell (*b*), laser-mediated catapulting of the single erythroblast off the microscope slide (*c*) and inspection of the resultant catapulted single cell as retrieved in the PCR cap (*d*).[.1]

reaction vessel which is conveniently located directly above the microscope stage (fig. 4). The genetic information is well preserved, as demonstrated by PCR results. This system is certainly easier to use than any of the other methods described above and, as a bonus, appears to have a higher efficiency for the transfer of single fetal cells than the use of microcapillary needles.

Future directions

Aspects which are currently receiving significant attention include the optimisation of WGA protocols [12, 46, 47, 67, 68] and modified multiplex PCR procedures [20, 43, 69]. The optimisation of both these procedures is very important as these allow the analyses of several loci. Unfortunately, present WGA procedures function best when using pools of cells. A further problem which needs to be overcome is that of ADO, which we have shown can occur during WGA procedures, such as PEP [21] and in multiplex PCR reactions [16]. Since ADO remains a significant problem when dealing with single cells, several strategies have been adopted to overcome this problem, including different cell lysis protocols, raising the denaturation temperature and more optimal thermostable polymerases [13, 71, 72]. The fact that these different protocols for cell lysis and PCR do affect ADO rates suggests that the cause of ADO might be more complex than just the result of template degradation [70].

Once these procedures have been perfected, it can be expected that this will open the door for more complex PCR analyses, such as those used in chip-array technology [73, 74]. In this manner it will be possible to screen several thousands of loci using the genetic starting material from a single cell, and thereby ushering in a new era of diagnostic possibilities.

Another exciting prospect is the ability to check for chromosomal aberrations in single cells by the aid of comparative genomic hybridisation (CGH) [12, 75, 76]. This technique permits not only the detection of chromosomal changes involving gross loss or gain of genetic material, such as aneuploidies and unbalanced translocations. In order to perform this complex assay on single of few cells, the groups of Wells [12], Jung [75] as well as that of Klein [76] have used WGA procedures to amplify the genetic material of single or pooled single cells until they yield sufficient quantities of DNA for this process. This development will no doubt soon be exploited in PGD [75] and in the analysis of micrometastatic tumour cells [76].

Even though these visions are currently far from reality, the use of SC-PCR is gaining in popularity on a daily basis, which means that numerous new applications for this technology are likely to be found. As such SC-PCR will no longer remain in the realm of a few hardcore PCR enthusiasts but will be used on a regular basis to answer new diagnostic and research-oriented questions.

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