SPANDIDOS PUBLICATIONS

New trends in non-invasive prenatal diagnosis: Applications of dielectrophoresis-based Lab-on-a-chip platforms to the identification and manipulation of rare cells (Review)

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Abstract. The isolation of rare cells, such as fetal nucleated red blood cells and trophoblasts, from maternal blood for non-invasive prenatal diagnosis is a new field of research exhibiting several difficulties since this strategy requires unresolved basic technological protocols for a successful outcome. However, several achievements in the field of Laboratory-on-a-chip (Lab-on-a-chip) technology have provided clear advancements in projects aimed at the isolation of rare cells from biological fluids. Among the most interesting approaches are those based on dielectrophoresis (DEP). DEP-based Lab-on-a-chip platforms have been demonstrated to be suitable for several applications in biotechnology and biomedicine. DEP-based arrays are able to manipulate single cells, which can be identified and moved throughout the DEP chip to recovery places. DEP buffers are compatible with molecular interactions between monoclonal antibodies and target cells, allowing integration of these devices with magnetic cell sorting (MACS). DEP treatment does not alter the viability of manipulated cells.

Contents

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1. Introduction

Current methods for the diagnosis of aneuploidy and monogenic disorders require invasive testing by amniocentesis, chorion villus biopsy or fetal blood sampling (1-4). These diagnostic techniques increase the frequency of fetal loss by ~0.5% (5). One alternative way for obtaining information on the gestating fetus involves recovery of fetal material from maternal blood (6). Currently, two independent approaches have followed this direction, one aimed at the identification and analysis of fetal DNA (7-13); the other aimed at the identification of circulating fetal cells, including trophoblasts (14-16), nucleated fetal red blood cells (fNRBC) (17-20), and nucleated white blood cells (21). Among these cell types, the most promising appears to be the identification and isolation of fNRBC and trophoblasts. In particular, trophoblasts have been recently described as very promising, considering the fact that, despite being rare within the blood of pregnant women, they can be grown *in vitro*.

Until recently, no established and routine methods for isolation of fetal cells from maternal blood have been available (22). Therefore, the development of technological platforms able to singularly manipulate rare cells is of great importance.

2. Intact fetal cells in maternal blood: A short history

In 1969, Walknowska *et al* (23) described Y chromatin in blood cells from women carrying male fetuses. Ten years later, recovery of fetal leukocytes from maternal blood by fluorescence-activated cell sorting (FACS) with antibodies against paternally derived HLA antigens was reported (24). Schmorl (25) described the deportation of fetal trophoblasts to the lungs of pregnant women suffering from eclampsia. Trophoblast cells have not found widespread application in diagnostic studies because they are rapidly cleared by the maternal pulmonary circulation and are likely to exhibit confined chromosomal mosaicism (26). Nucleated red blood

cells (NRBC) are the most common cells in fetal blood during early pregnancy. Since they have a relatively short half-life and express hematopoietic plasma membrane antigens, such as the transferrin receptor (CD71) and the glycophorin A cell surface molecule and intracellular markers (ε and γ globin chains), fetal NRBC have become the targets of choice. In 1990, Bianchi *et al* (27) recovered fetal NRBC in maternal blood by using FACS. Fetal origin of the separated cells was confirmed by the presence of Y-chromosome DNA, corresponding to the sex of the fetus.

3. Isolation of intact fetal nucleated red blood cells in maternal blood: The state of the art

Although the most represented fetal cells, fNRBC, are rare in respect to maternal cells, they can be enriched in maternal blood samples by using antibodies against specific fetal antigens or by physical methods such as gradient or electrophoretic mobility-based separations. Antibodies have been used with fluorescence-activated cell sorting (FACS) (28) or magnetic-activated cell sorting (MACS) (29). These methods exploit antigenic differences between cells. FACS is able to enrich cells with high purity so that slides with sorted cells can be readily scanned manually. It also allows multi-parameter sorting and can be adapted for use with intracytoplasmic antigens. MACS, on the other hand, using magnetic beads coated with specific antibodies, is a faster bench-top technique better suited to process larger cell numbers. Both negative and positive selections can be performed on the same population of cells. Detection of levels of fetal cells with these two methods is however difficult in relation to the small numbers of circulating fetal cells and the loss of fetal cells during the enrichment procedures. Bischoff *et al* (30) reported a simple and rapid-density-based progenitor cell enrichment approach. The samples were labeled with a RosetteSep™ progenitor antibody cocktail to remove unwanted maternal white cells (mature T-cells, B-cells, granulocytes, natural killer, neutrophils and myelomonocytic cells). The cellular fraction collected was analyzed by either fluorescent *in situ* hybridization (FISH) or real time-PCR for the presence of intact fetal cells and to quantify Y-chromosome-specific DYS1 sequences, respectively. The accuracy of detection rates of the progenitor enrichment approach were 53-89%, and fetal sequences were detected in the range 0.067-1.167 genome equivalents per milliliter of blood. This method targets progenitor cells that are not necessarily of the erythroid lineage and may also allow expansion in culture and characterization of the fetal cell types that circulate in maternal blood. Some lymphocytes are long lived, and with this approach there is concern that enriched progenitors may be the vestiges of previous pregnancies and do not represent the true fetal genetic status of the current pregnancy (30). Charge flow separation (CFS) is an antibody-independent selection method of fetal cells that relies on the behaviour of cells in an electric field and a buffer counterflow gradient (31). It is an automated rapid method that purifies NRBC from maternal blood, and ~30% of these cells are fetal. For non-invasive prenatal diagnosis, NRBC from the fetus must be precisely identified. They are able to be distinguished from maternal cells by identifying paternal DNA sequences (32).

Table I. Antibodies used for NRBC isolation.

Antibody/target	Reference
CD71	Winichagoon P *et al*, 2005 (44) Nagy GR *et al*, 2005 (45) Christensen B *et al*, 2005 (6) Al-Mufti R *et al*, 2004 (47) Al-Mufti R *et al*, 2004 (48) Al-Mufti R *et al*, 2000 (59) Furusawa T *et al*, 1998 (66) Navenot JM *et al*, 1997 (68) Savion S *et al,* 1997 (69)
2F6.3	Fernandez A *et al*, 2005 (46)
HbF	Kazama A *et al*, 1996 (70)
CD45/CD14	Christensen B *et al*, 2005 (6) Wang Z and Liebhaber SA, 1999 (65)
Glycophorin-A	Collarini EJ *et al*, 2001 (52) Choolani M *et al*, 2001 (43) Sekizawa A *et al*, 1999 (64) Ziegler BL *et al*, 1995 (71)
CD45	Martel-Petit V *et al*, 2001 (55) Jakobs ME *et al*, 2000 (57) Di Naro E *et al*, 2000 (61) Cunningham J *et al*, 1999 (63)
γ, ε and ζ hemoglobin chains	Jackson L *et al*, 1993 (5) Christensen B *et al*, 2003 (49) Christensen B *et al*, 2003 (50) Collarini EJ *et al*, 2001 (53) Samura O *et al*, 2000 (58) Sekizawa A *et al*, 1999 (62) Mavrou A *et al*, 2003 (17) Xu H *et al*, 2001 (54)
Glycophorin-C	Choi JW *et al*, 2002 (51)
Embryonic hemoglobin (HbE)	Sekizawa A *et al*, 2000 (56)
ζ hemoglobin chains	Al-Mufti R *et al*, 2000 (60)
FB3-2; H3-3; 2-6B/6	Pezzolo A *et al*, 1997 (67)

This method can be extended using micromanipulation of candidate fetal cells and amplification of chromosome-specific short tandem repeats (33). It is under investigation whether recovery of fetal cells with CFS is higher than that obtained with other separation methods. The most important molecular techniques that have allowed genetic analysis of enriched fetal cells are PCR and FISH. The ability of PCR to amplify minute quantities of DNA (even a single copy) >1 billion-fold has been exploited for the prenatal diagnosis of monogenic disorders from maternal blood (34-36). Chromosomal FISH allows the detection of aneuploidy and chromosomal rearrangements in interphase nuclei. It has been used to detect most of the major fetal aneuploidies within fetal cells isolated from maternal blood (28,37-39). Fetal traits which have been identified so far among the separated fetal cells include blood group antigen, the common trisomies,

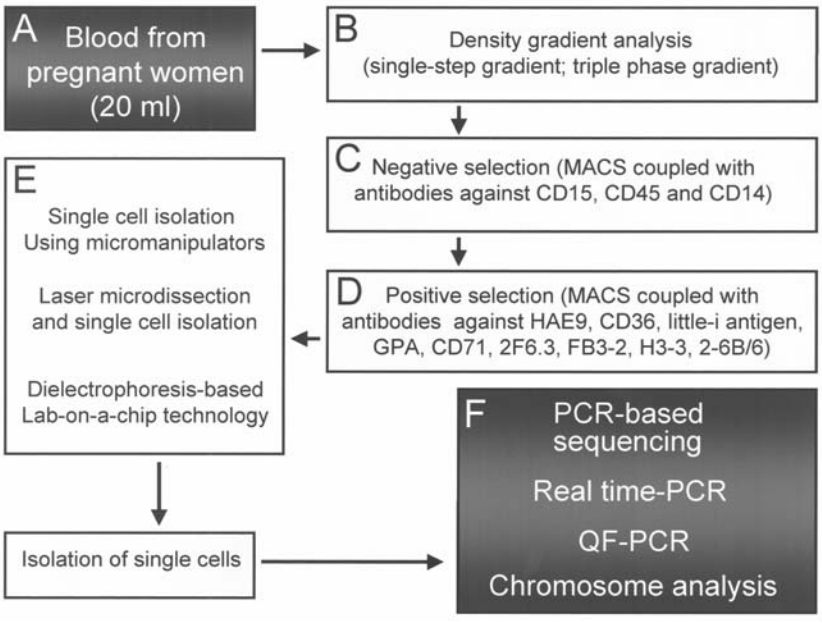

Figure 1. Flow chart depicting the strategy for enrichment of fetal cells from maternal blood for diagnostic purposes.

triploidy, polymorphic DNA repeats, and some single-gene disorders (34,39-42). All of these methods result in the enrichment of fetal cells among larger populations of maternal cells, but they do not enable recovery of pure populations of fetal cells. Experimental approaches which combine fetal cell identification with molecular genetic diagnosis with *in situ* techniques circumvent these limitations and are especially suited for automation (43). Unfortunately prenatal diagnosis from maternal blood is not practicable for ~30% of pregnant women, because the NRBC are not recovered. It is then important to optimize enrichment identification end diagnostic protocols.

A partial list of antibodies used for NRBC isolation is provided in Table I.

4. Isolation of trophoblasts in maternal blood: New trends

The relevance of trophoblasts as target cells is under debate. Among the identified drawbacks are the following: a) trophoblasts are long-term circulating cells and, therefore, may originate from previous fetuses; b) trophoblasts; c) a high level of heterogeneity in the number of trophoblasts/ml of maternal blood has been described. However, recent advances in the isolation of trophoblasts from maternal blood have demonstrated that the potential application of these cells in non-invasive prenatal diagnosis is high (72). Trophoblast cells are isolated by several methods related to surface antigen expression (e.g. expression of HLA-G) and cell size. The idea of trophoblasts as target cells has been rethought from a point of view that has not yet been pursued; the potential to grow *in vitro* under suitable experimental conditions, thereby improving fetal cell detection and, possibly, allowing diagnostic approaches based on metaphase chromosome analysis. Accordingly, the HLA-G-positive fraction of sorted cells can be sub-cultured, greatly facilitating studies in molecular biology.

5. In the search of a partner for MACS and FACS: Theory and biotechnological applications of dielectrophoresis (DEP)

All the available information indicates that MACS technology allows the collection of cell populations significantly enriched in fetal erythroblasts or trophoblasts (19,30,73-75). A combination of the MACS procedure with other strategies for isolation of rare cells is highly needed to obtain almost a pure cell population. Up to now, an integrated system has not been available.

In Fig. 1, a possible flow-chart is depicted allowing enrichment of fetal cells from maternal blood for molecular or cytogenetic analysis. There is general agreement that at least 20 ml of blood must be obtained in order to ensure pure cell populations for analysis (Fig. 1A). After density gradient separations (Fig. 1B) and negative selection (Fig. 1C) the cells of interest are partially purified using antibodies to surface antigens recognizing fetal cells (Fig. 1D). This step does not allow a purification of pure cell populations since most of the antibodies used recognize and co-purify adult cells. Therefore, further analyses should be proposed, based on the labeling of fetal cells on the one hand and on the manipulation of single cells on the other. Concerning the latter step, several approaches have been proposed including single-cell isolation using micromanipulators, laser microdissection and single-cell isolation and dielectrophoresis-based Lab-on-a-chip technology (Fig. 1E). After isolation of single cells, several molecular biological techniques have been described for molecular characterization of the fetal cell genome, including PCR-based sequencing, real time-PCR, and Quantitative Fluorescence PCR (QF-PCR) (Fig. 1F). In case the procedure allows further cell culturing steps, chromosomal analysis is feasible (Fig. 1F). Among the different technologies recently proposed for isolation of rare cells from a mixed population, this review focuses on dielectrophoresis (76).

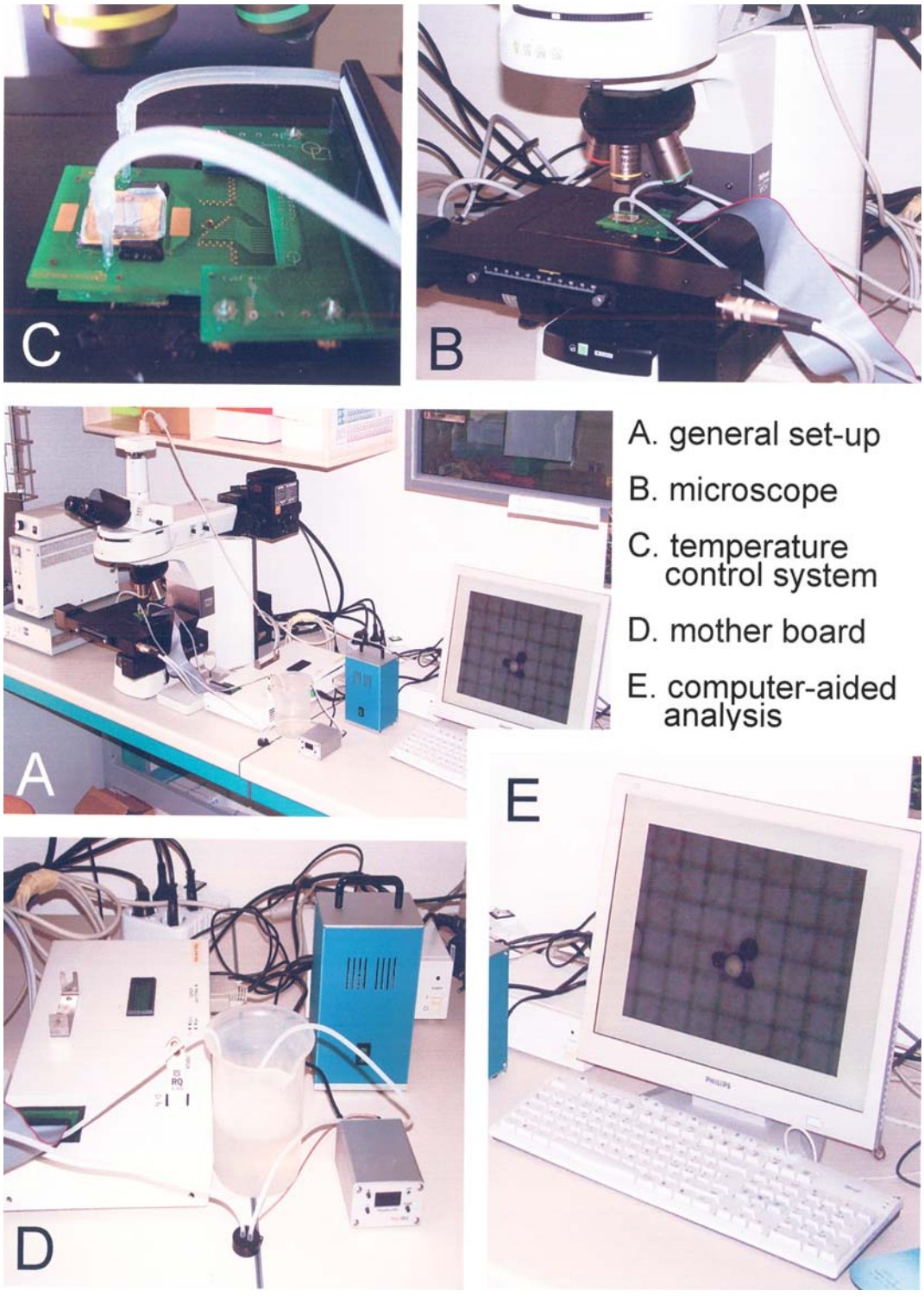

Figure 2. (A) Set-up of the Lab-on-a-chip assembly, consisting of a microscope (B), a micropump for temperature stabilization (C), a mother board (D), and a personal computer (E). Modified from Borgatti *et al* (97).

DEP is the movement of particles in non-uniform electric fields (77,78). Charges in the particle itself are not necessary for the effect to occur. This is due to the fact that when an electric field is applied to a system consisting of particles suspended in a liquid, a dipole moment is induced, due to the electrical polarizations at the interface between the particle and the suspending liquid (77-83). If the field is non-uniform, the particles experience a translation force (DEP force) of magnitude and polarity, depending not only on the electrical properties of the particles and the medium, but also on the magnitude and frequency of the applied electric field. This means that for a given particle type and suspending medium, the particle can experience, at a certain frequency of the electrode applied voltages, a translation force directed towards regions of high electric field strength (this phenomenon is called pDEP). Alternatively, by simply changing the frequency,

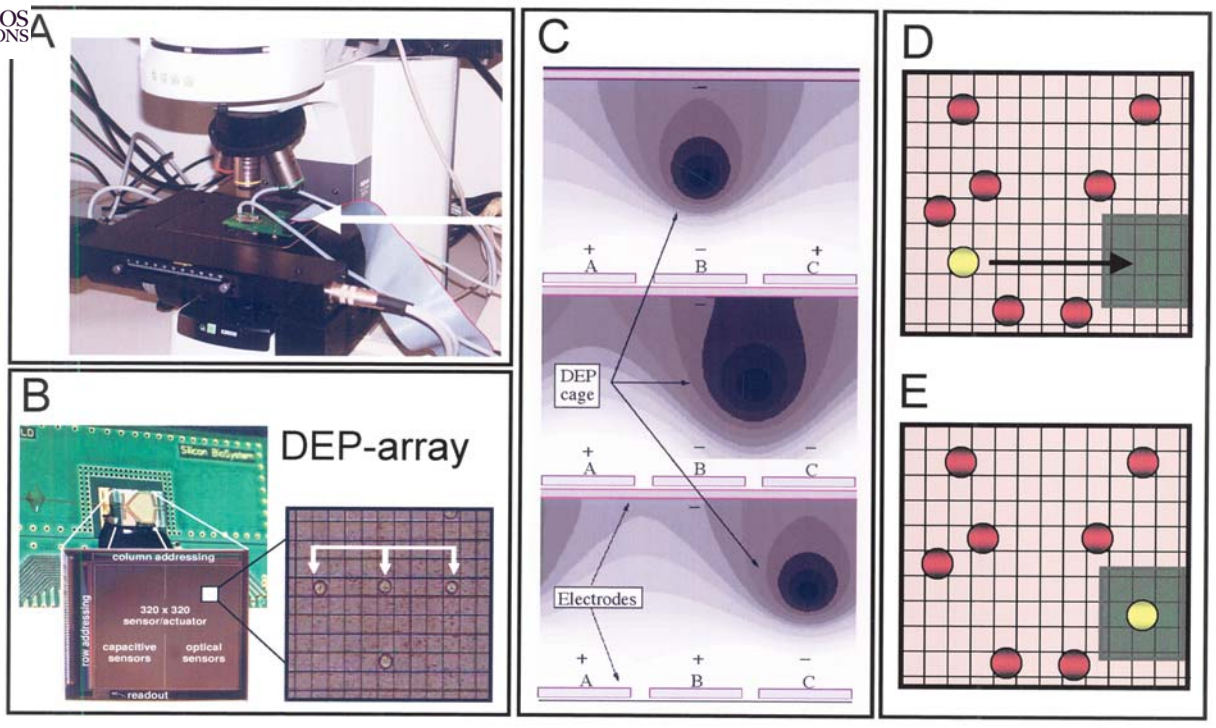

Figure 3. (A,B) Detail of structures of the DEP-array. The location of the device under the microscope is indicated with an arrow in panel A, while the structure is shown in panel B. In the insert of panel B, single cells are entrapped within DEP-cages and separated. (C) Simulation of the movement of a single spherical DEP-cage along the DEP-array. (D,E) Programmed routing of an identified single cell (yellow) to a recovery field (green). Panels A-C are modified from Borgatti *et al* (91).

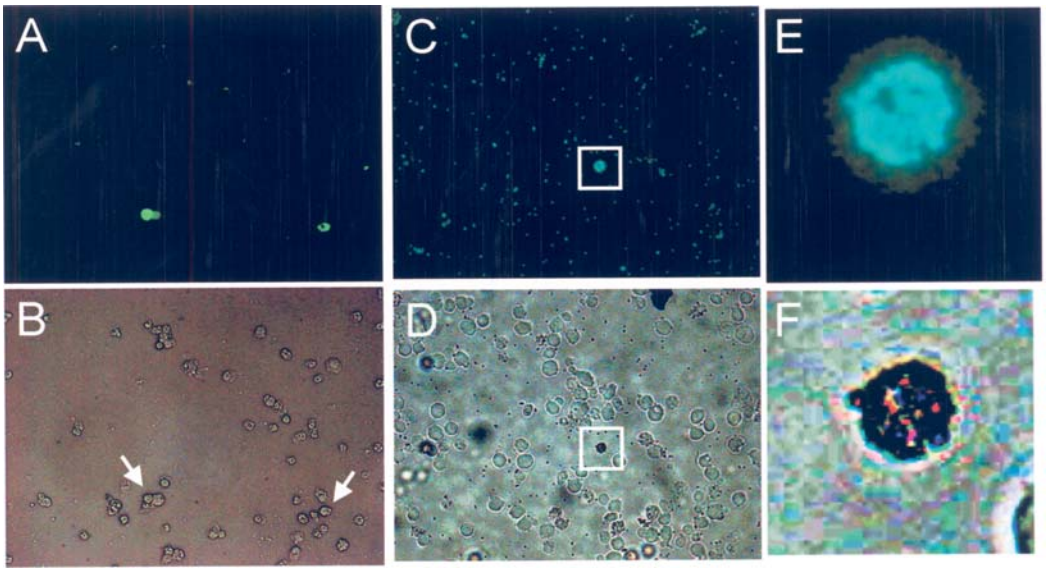

Figure 4. Identification of γ-globin-containing (A,B) and MEM-G9-positive (C-F) cells. Staining with fluorescent anti-γ-globin antibodies is a strategy to identify fNRBC (18); binding of MEM-G9-loaded fluorescent microspheres are currently employed to detect HLA-G-positive trophoblasts. A and C, microscopic analysis; B and D, fluorescence analysis. E and F are enlargements of panels C and D (the enlarged region is identified by a box).

the particle may experience a force that will direct it away from high electric field strength regions (this phenomenon is called nDEP).

The general set-up of a DEP-based system is shown in Fig. 2A, and is generally constituted by a microscope (Fig. 2A), connected with CDD-camera and computer (Fig. 2A and E), and platforms suitable for inclusion of the Lab-on-a-chip devices (Fig. 2C). Several DEP-based Lab-on-a-chip devices were recently described and found to be suitable for biotechnological applications in the field of isolation of single-cell population as well as manipulation of single biological objects, including cells and microspheres.

6. DEP-based Lab-on-a-chip devices with high-density arrayed electrodes

Fig. 3A and B shows the DEP-array, which is constituted by a microchamber defined by the chip surface and a conductive-

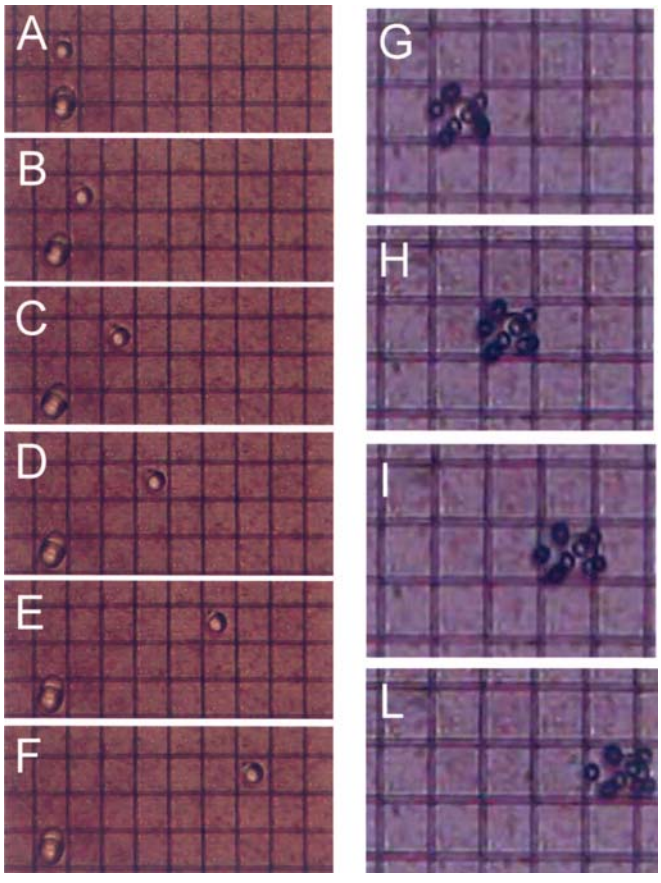

Figure 5. Routing of a single cell (A-F) or a cell-microsphere complex (G-L). Photographs were taken at the beginning (A,G) or after 30 sec (B,H), 1 min (C and I), 90 sec (D and L), 2 min (E) and 2.5 min (F). These data are taken from Borgatti *et al* (91).

glass lid. The chip surface implements a two-dimensional array of 320x320 microsites, each consisting of a superficial electrode, embedded sensors and logic. The electrode array is implemented with CMOS (complementary metal oxyde semiconductor) top-metal and protected from the liquid by the standard CMOS passivation. The design, technical parameters, building approach and manufacture of this DEP-based arrayed device have been described by Medoro *et al* (84-86) and Manaresi *et al* (87). In this system, a closed DEP-cage in the spatial region above a microsite can be created by connecting the associated electrode and the microchamber lid to a counter-phase sinusoidal voltage, while the electrode of the neighboring microsites is connected to an in-phase sinusoidal voltage. A minimum field is thus created in the liquid, corresponding to a DEP-cage in which, depending on its size, one or more particles can be trapped and levitated. This system allows the creation of 4,000-10,000 DEP-cages, able to entrap single cells or cell-microsphere complexes (85,86). By changing, under software control, the pattern of voltages applied to the electrodes, these spherical DEP-cages can be independently moved around the device plane (Fig. 3C), thus grabbing and dragging cells and/or microbeads across the chip. Particles in the sample can be detected by the changes in optical radiation impinging on the photodiode associated with each microsite. Thanks to the small pitch of the electrodes, single cells can be individually trapped in separate cages (insert, Fig. 3B) and independently moved on the device (Fig. 3C and in the representative experiments depicted in Fig. 3D and E). Particle position is digitally controlled step-by-step in a deterministic way, by applying a corresponding pattern of voltages to the array which set the position of the DEP-cages.

The most important features of the Lab-on-a-chip device are: a) it carries out functions of both actuating and sensing

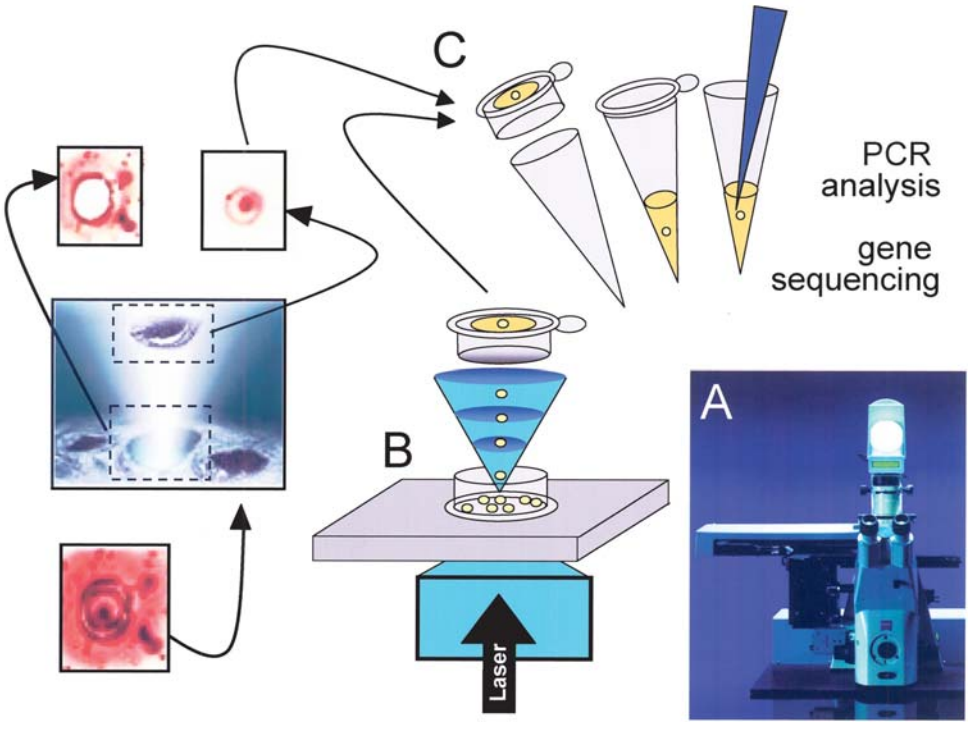

Figure 6. Scheme outlining the Leica PALM MicroBean system; (A) the instrument and (B) the concept of laser pressure catapulting. After catapulting, the isolated cells can be recovered for molecular diagnosis (C).

he DEP-chip is optimal for the separation of large of eukaryotic cells; c) the DEP-array can be programmed for moving single cells; and d) single cells can be forced to contact antibody-exposing microspheres.

In respect to isolation of a pure cell population, additional published results (reviewed in ref. 84) have firmly demonstrated that human cell populations of different histotype and differentiation stage are able to be isolated using DEP-based devices. These features propose Lab-on-a-chip platforms for diagnostic applications, cell separation and characterization in the field of non-invasive prenatal diagnosis (88-98).

First, in order to follow the step indicated in Fig. 1C and D, it is crucial to demonstrate that interaction between antibodies and surface antigens is stable in buffers employed for DEP. This is shown in Fig. 4A and B, showing staining of γ-globin-expressing cells (expressed by fNRBC) in 280 mM mannitol and 6.25 mM KCl, a buffer commonly used in DEP experiments. In addition, cells can be labeled with fluorescence beads carrying specific antibodies. In Fig. 4C-F a single HLA-G-expressing cell is identified in 280 mM mannitol/6.25 mM KCl from the other negative cells by using the MoAb MEM-G9, specific for trophoblasts.

Secondly, after identification of single cells or cell-microsphere complexes, these can be forced to move to pre-identified areas of the Lab-on-a-chip device. This is demonstrated in the representative experiment shown in Fig. 5, in which a single cell (A-F) or a single-cell microsphere complex (G-L) are moved along the chip, allowing the concentration of the identified cells to recovery places of the device, as indicated by panels D and E of Fig. 3.

The data presented here and those available in the literature indicate that the dielectrophoresis (DEP)-based Lab-on-a-chip approach meets the required criteria for the optimization of enrichment of fetal cells in the blood of pregnant women for non-invasive prenatal diagnosis.

7. Competing technologies: Noncontact laser microdissection and pressure catapulting (LMPC) for isolation of single cells

In recent years, laser microdissection and pressure catapulting (LMPC) has been described as an emerging technology for the isolation of identified cells for genomic analysis (99-101). This approach enables pure and homogeneous sample preparation (102,103). For microdissection, the force of focused laser light is used to excise selected cells or large tissue areas from object slides or from living cell cultures down to a resolution of individual single cells and subcellular components like organelles or chromosomes, respectively (Fig. 6A and B). After microdissection this sample is directly catapulted into an appropriate collection device (Fig. 6B). As the entire process is conducted without any mechanical contact, it enables pure sample retrieval from a morphologically defined origin without cross-contamination. Wherever homogenous samples are required for subsequent analysis of, e.g. cell areas, single cells, or chromosomes, the PALM MicroBeam system is an indispensable tool. The integration of image analysis platforms fully automates screening, identification, and finally subsequent high-throughput sample handling. These samples can be directly linked into versatile downstream applications, such as single-cell mRNA extraction, different PCR methods, microarray techniques, and various others (Fig. 6C).

8. Conclusions

The optimization of prenatal diagnosis for chromosomal and monogenic disorders can be greatly facilitated if obtaining fetal material from maternal blood is efficiently and reproducibly obtained, avoiding the risks associated with amniocentesis, chorionic villus sampling and fetal blood sampling.

The isolation of rare cells from maternal blood for non-invasive prenatal diagnosis is a new field of research exhibiting several difficulties since this strategy requires several basic technological protocols for a successful outcome (19,45,46,48, 50,52,62,68,104-112).

Risk factors for DEP-based Lab-on-a-chip development are as follows. i) Antigen-antibody interactions in the DEP buffers. The binding between monoclonal antibodies and target cells occurs in mannitol-based buffers. However, the present published data do not support the hypothesis that for all the monoclonal antibodies necessary for fetal cell identification in non-invasive prenatal diagnosis, the efficiency in DEP buffer is the same of binding reactions performed in RPMI. ii) Isolation of cells after DEP-based Lab-on-a-chip separation. The isolation of cells from the DEP-array, despite not being optimized and published so far, is expected to be developed for FACS/MACS and Lab-on-a-chip enrichment of fetal cells from maternal blood. iii) Vitality of the cells after DEP-manipulation and Lab-on-a-chip separation. This point is not crucial for DNA-based diagnosis (performance of PCR, microsequencing, allele discrimination); however, it might be of great interest for chromosomal analysis. The collective data suggest that no mutations are introduced by DEP treatment, and cells can be viable after DEP separation.

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