Micro tools for cell handling

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ABSTRACT

Microfabrication had a major impact on electronics and is expected to have an equally pronounced effect on chemistry and life sciences. Exploitation of these scientific fields is becoming increasingly dependent on the availability of systems that can perform fast and accurate analyses, using minute volumes of sample. By combining microfluidics with micromechanics, microoptics, and microelectronics, systems can be realized that perform complete analyses. The possibility to realize structures with sizes that are in the same range as biological cells makes microtechnology especially interesting for cell analysis. Cell analysis already forms an important, integral part of medical diagnostics and research. Microtechnology provides the opportunity to refine existing cell analysis tools but also allows fabrication of instruments that cannot be realized with conventional technologies. Examples of first steps along this path are provided.

Keywords: Microsystem technology, μTAS, Lab-on-chip, Microfluidics, Integrated optics, Cytometry, FACS

1. INTRODUCTION

Biological cells represent the most basic unit of life that mediate all functions necessary to build and maintain tissue. Any disease state ultimately reflects corruption of one or more cell functions. This key role of cells has fuelled the development of a variety of instruments for the analysis of cells including microscopes, cytometers, fluorescent activated cell sorters (FACS), and Coulter counters. By providing the scientific and medical community with information about cell concentration, cell morphology, and cell phenotype these devices have significantly improved medical diagnostics and provided new approaches to medical therapy. Conventional instruments are usually large, heavy, expensive, and are operated by trained personnel. They require relatively large sample volumes and are difficult to sterilize. Following international trends and satisfying ever increasing demands from the research and medical community, equipment for cell analysis needs to be further refined. Taking into account the need for high through-put, point-of-care (POC), smaller sample volumes, lower reagent consumption, single use systems, increased functionality, and single cell analysis, microsystem technology (MST) offers an interesting technology platform to fulfil these needs.

MST has played a pivotal role in the succes of microelectronics. The micromachining of semiconductor materials by means of photolitography made it possible to miniaturize electronic components. Miniaturization in turn allowed for integration of components with different functionalities and hence complex processing of electronic signals on chip. The strategy of miniaturization and integration of electronic components offers portability, reduced power consumption, faster performance, increased functionality, and increased reliability. Almost in parallel with the development of microelectronics, mechanical structures were miniaturized yielding e.g. accelerometers for airbag deployment that are now widely used commercially in cars[1]. In the late seventies the first microfabricated chemical analysis system employing microfluidics was reported[2]. Examples like these show that the advantages of miniaturization and functional integration are not limited to electronics but can actually be expanded to other disciplines. In 1990 Manz and Widmer coined the concept of micro Total Analysis Systems (μTAS), systems where all component stages of analysis like sample pre-preparation, chemical reactions, analyte separation, analyte purification, analyte detection, and data analysis are performed in an integrated and automated fashion. The realization of such analysis systems requires miniaturization and integration of a wide variety of components, e.g. mechanic, fluidic, optic, and electronic. The concept of μTAS can also be applied to cell analysis where the necessary analysis steps are performed automatically in a microsystem.

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2. RESULTS AND DISCUSSION

2.1. Micro Conductive Particle Counting

Counting and sizing of biological cells has been used for half a century as a diagnostic tool. Different instruments involving optics and electronics are used for counting and sizing of particles or cells where many of these instruments require precision fluidic dispensing, a laser and a sensitive detection system. Coulter counting provides a means of counting and sizing cells without the need for expensive or complex equipment[3]. Despite the limitations of this instrument to counting and sizing of cells or particles only, there are many examples where this basic information is sufficient for diagnostics. The principle behind Coulter counting is based on conductivity measurements where cells or particles are injected into an electrolyte and aspirated through a small aperture in a membrane that separates two electrically isolated chambers (Figure 1A). When a cell or particle passes through the aperture the resistivity over the membrane rises. Traditional Coulter counters rely on apertures obtained by mechanical machining. Silicon micromachining allows the fabrication of apertures that are much more accurate and refined. The realization of Coulter counting in a microsystem also opens up the possibility to produce cheap and portable cell/particle counters that can be used for POC. Ultimately, microconductive particle counting can be integrated with other functional components as to arrive at total cell analysis systems.

The Coulter counting principle is implemented in a simple silicon chip-based particle counter. The instrument is comprised of a transducer with two chambers only interconnected by fluidic and electrical means through the silicon aperture (Figure 1B). Fabrication of silicon apertures is straightforward: The aperture is anisotropically etched by reactive ion etching in the front of the wafer whereafter the wafer is etched from the backside in KOH to make a 5 by 5-millimeter membrane around the aperture (Figure 1C). Finally the wafer is oxidized for electrical isolation and cut into small 1 by 1 cm chips. The shape of the aperture is controlled to guarantee the formation of a homogeneous electrical field in the middle of the aperture. This homogeneous field in the aperture is essential to ensure good correlation between pulse height and cell volume.

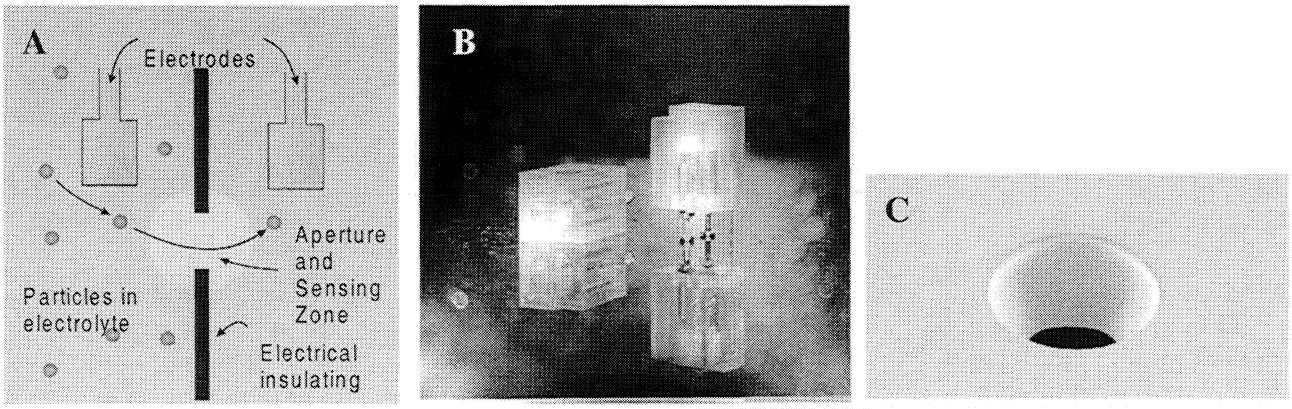

Figure 1: A) Schematic drawing of the principle behind Coulter counting and micro conductive particle counting. B) Polymer cartridge containing the silicon transducer with aperture. C) scanning electron microscope image of a 30 μm silicon aperture.

One of the most important but also rather critical measurements to make with the micro conductive particle counter is the determination of concentrations of particles or cells. In order to get accurate concentration readings the particle suspension has to be homogeneous, the numbers of counted particles must be high enough and the amount of liquid going through the aperture must be accurately controlled. Furthermore, in association with the collection of data on a computer, sample rate and trigger level must be carefully considered. One of the more intrinsic problems is the occurrence of clusters of particles, whereof the most evident are doublets and triplets. These are most frequent with non-biological particles such as latex beads. In figure 2 the size distribution of latex beads obtained with a 30 μm silicon aperture is shown. The beads are 9.146 μm with a standard deviation of 0.577 μm. The doublets are double in volume, thus resulting in a mean diameter of 11.5 μm and in the same way triplets result in a mean diameter of 13.2 μm. Since the Gaussian distribution curves from single,

doublets, and triplets overlap, the exact number of single particles cannot be easily calculated, and some interpretation must be done. In this case approximately 20% of the counts are doublets and approximately 10 % are triplets. This gives a multiplication factor to the number of counted particles of 1.4. The counting experiment is run three times with a total volume of sample of 50 µl for each run, which gives a mean count of 11,384 particles, and a standard deviation of 389 (3.4%). With the correction factor of 1.4 this gives a total concentration of: $319 \cdot 10^3 \pm 11 \cdot 10^3$ particles/ml. Control experiments, where small amounts of sample were taken from the counting chamber and counted by eye under a microscope, yielded a concentration of $314 \cdot 10^3 \pm 25 \cdot 10^3$ particles/ml which is in excellent agreement with the experimental data.

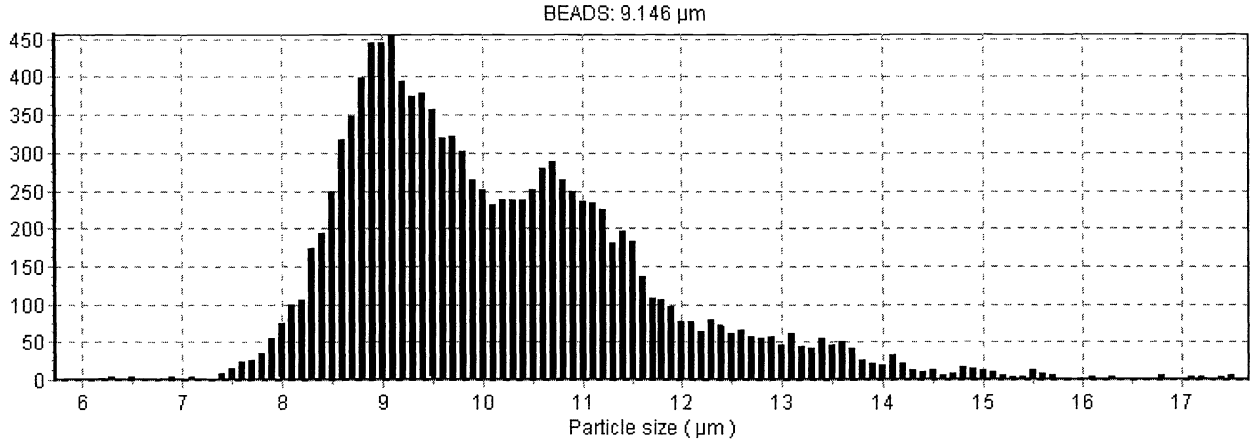

Figure 2: Histogram showing singles, doublets and triplets of 9.146 μm latex beads counted in a 50 µl sample at a sampling rate of 100 µl/min.

One example of a problem that can benefit from a portable, simple and cheap cell counter is the quality control of milk. Milk quality is essential for farmers, dairy producers and consumers. Farmers are obliged to deliver milk with a certain minimum quality which is normally reflected and controlled by the Somatic Cell Count (SCC). SCC-levels increase with changes in diet, stress or udder infections (mastitis). To keep the SCC level low, the farmer must show great awareness towards hygiene in respect to his cows. The price paid for the milk by the dairy producer depends on the SCC-level where high SCC-levels lower the price per unit volume. A particle counter as described here can help farmers and dairy producers to get control over SCC-levels.

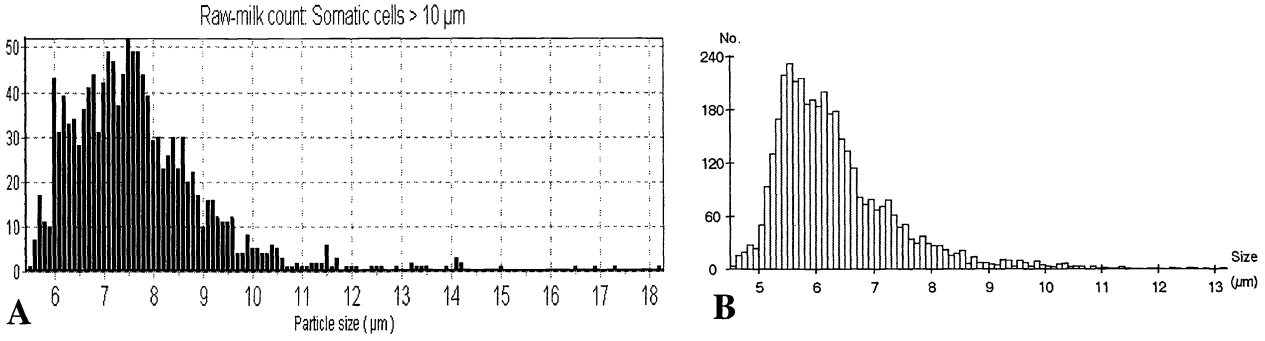

Figure 3: A) Size distribution of particles in fresh milk. The most dominant feature is fat globules with mean sizes between 5 and 8 µm. Somatic cells are significantly larger and can be found above 10µm. B) Size profile of unprocessed human blood. The profile is dominated by red blood cells with a mean size around 6 µm.

In contrast to the current methods where milk from different cows is pooled, cows can be individually tested. Samples of milk are obtained through the laboratory of the Danish Diary Board in Ladelund (Denmark). SSC-levels in these samples were measured in Ladelund with the commercially available Fossomatic cytometer (Foss – Hillerød, Denmark) as a reference. Milk contains a large number of fat mono-globules of 5-8 μm size, which are evenly distributed in fresh milk. As somatic cells are larger then the fat mono-globules, SCC numbers can be derived by size exclusion and further signal processing. Alternatively, fat globules can be chemically removed from the sample. Figure 3A shows a typical result of a milk sample counted with the micro conductive particle counter. In comparing these experimental results to those obtained with the Fossomatic instrument a 100% correlation on identifying cows with mastitis is achieved.

Counting cells is by no means limited to the control of milk quality but is equally important in medical diagnostics and research. This wider applicability is demonstrated in figure 3B which depicts a human blood cell count. The laboratory prototype that yielded these results is being commercialized by Chempaq (Lyngby, Denmark). A new generation cell/particle counters is under development that will benefit of functional integration of the micro conductive cell counting with other components that are important for cell analysis.

2.2. Cytometry and Cell Sorting in Microfluidic Structures

Cytometry and FACS, the analysis and isolation of specific cell populations by marking with fluorochromes, are the most popular cell analysis tools today. Central to these instruments are components for the handling of liquids and optical detection[4]. Typically, cells are coaxially sheathed with a buffer and led single file through a laser beam. Scattered laser light and emission light are collected and measured by means of a photo multiplier tube. Miniaturization of cytometers and FACS instruments offers a number of advantages over conventional equipments, e.g. better performance because of liquid handling in the laminary flow regime, closed systems reducing the risk of infection of the sorted cells and reducing potential biohazard risks of working with infectious materials, improved efficiency, recovery and purity of sorted cells, POC instruments, and single use devices. The most important advantage of microsystems however is the possibility to integrate various functional modules on the same chip, improving overall performance. Such functional modules may include components for automated cell staining, holding and cultivation chambers for sorted cells, PCR amplification, and microarrays for genetic analysis.

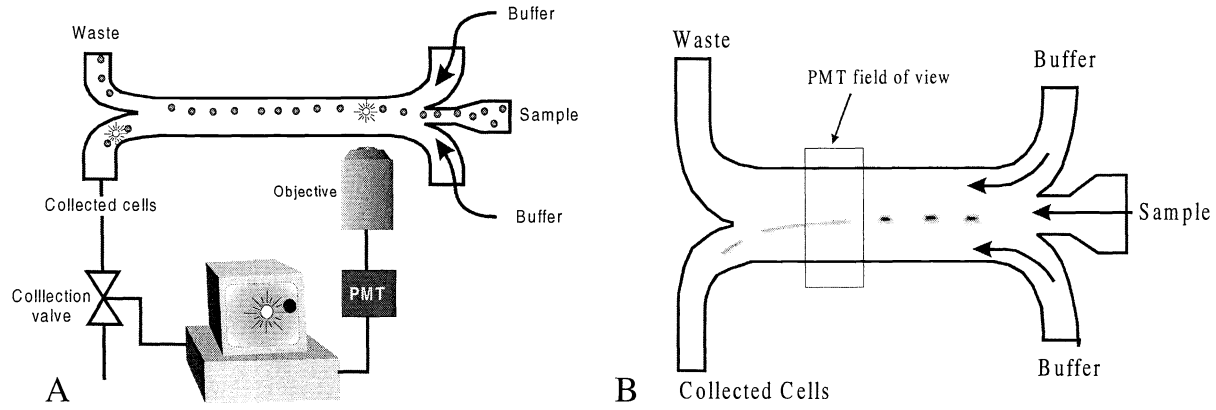

Figure 4: A) Schematic depiction of the principle for fluorescent activated cell sorting in a microfluidic structure. B) 5 Inverted frames of a VCR recording of a fluorescent activated sorting experiment are super imposed on a schematic representation of the microfluidic sorter structure. The fluorescent particle enters the structure, is detected by the PMT, and forced into the collecting outlet. During switching of the valve most of the liquid exits the structure through the collecting outlet. The particle is accelerated and produces a comet-like appearance.

One of the diagnostic areas where traditional approaches to cell sorting has been shortcoming is rare event cell sorting. An example where rare event cell sorting is of great importance is prenatal diagnostics based on fetal cells circulating in the

peripheral blood of pregnant women in the first 3 months of pregnancy. The procedures that are currently available for prenatal diagnostics are invasive and carry around a 1 % risk of pregnancy loss. Therefore, prenatal diagnostic is only offered to women with increased risks for carrying a fetus with genetic abnormalities.Several investigators have reported the presence of fetal cells in maternal peripheral blood at an estimated frequency of about 10 to 100 fetal cells per 1 ml full blood. If these cells can be isolated from the maternal blood they can be used for prenatal genetic diagnosis without the risk of pregnancy loss. Conventional equipment is limited by low speed, high dilution, sterility problems and low recovery of the desired cells. It is our aim to develop and adapt microsystem technology to enrich fetal cells from maternal blood.

Microfluidic components are etched in a silicon wafer by reactive ion etching. After processing, the wafer is anodically bonded to a Pyrex glass plate, which allows optical detection and external observation of the sorting process. The individual microstructures are diced and mounted in a metal holder. Fluid interconnections are made from the back by through holes.

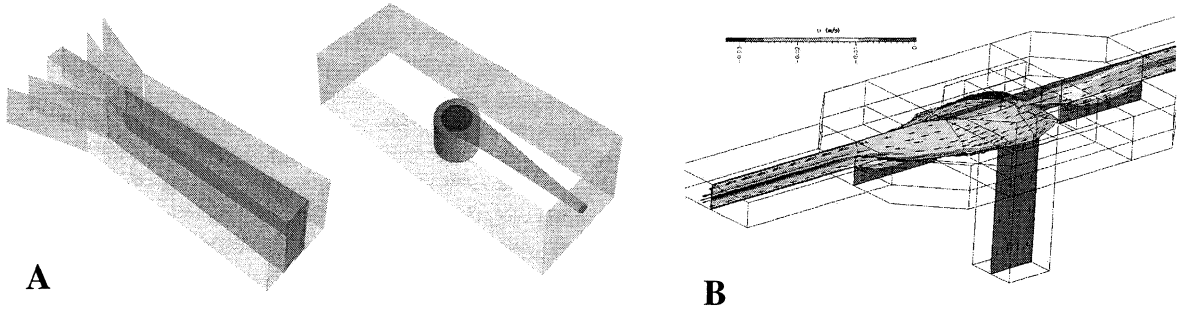

Figure 5: A) Sample inlet in old and new design. In the old design (left) the sample is only sheated from the flanking sides but not from the top or bottom. In the new design (right) the sample is introduced in the buffer stream through a "chimney" providing single step coaxial sheating. B) Computer simulation of the performance of the "chimney" structure.

Figure 4A shows a schematic depiction of a typical hybrid set-up for cell sorting in a microstructure. Pumping, valving, and optical detection are performed off chip. In this sorter structure particles, labeled with a fluorochrome and sheathed by two buffer streams, are excited by a laser and detected by a photo multiplier tube (PMT). Maintaining a slight overpressure on the waste outlet, particles of interest are forced to the collecting outlet by opening the collection valve which is triggered by the PMT signal (Figure 4B).

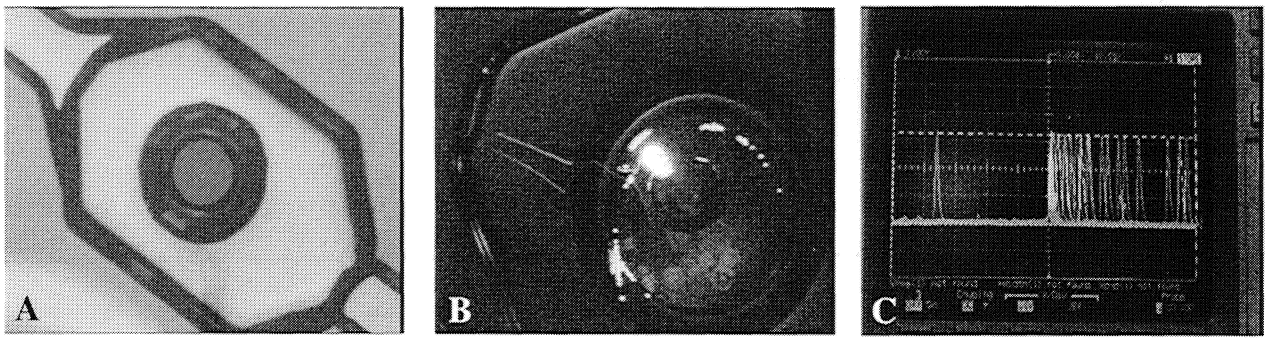

Figure 6: A) "Chimney" structure for single step coaxial flow realized in silicon by reactive ion etching. B) VCR recording of fluorescent beads passing through the "chimney" inlet. C) Osciloscope visualization of the PMT signal: Even distribution of peak height due to even distribution of particle speed in the "chimney" structure.

To demonstrate rare event sorting we used 10-fold diluted EDTA stabilized chicken red blood cells (CRBC) (Statens Serum Institute, Copenhagen) mixed with 10 μm fluorescent latex beads (Polysciences Inc, Warrington PA) to final concentrations

of $2.6 \cdot 10^3$ beads/ml and $1.1 \cdot 10^8$ CRBC/ml, respectively. To shorten the analysis time the sheathing of sample was adjusted such that multiple cells passed the detection window simultaneously. First tests resulted in 100-fold enrichments at sampling rates of 10,000 cells/s. Although these experiments demonstrated the feasibility of performing rare event cell sorting in a microstructure they also pointed at weaknesses that had to be dealt with in order achieve the ultimate goal of fetal cell sorting.

In the first generation microfluidic cell sorters sample is only sheathed on the two flanking sides but not on the top or bottom. This incomplete hydrodynamic focusing has a number of disadvantages. Fluorescent signals from beads with the same amount of fluorochrome show great variation depending on whether the beads are in the focal plane or not. Furthermore, as a result of laminar flow, the speed of the cells or particles moving through the structure depends on the position of the cell or particle in the channel. In response, we developed a component for coaxial sample sheathing that fulfilled our critera of single step coaxial sheathing, simple silicon processing and monolithic integration. The resulting "smoking chimney"[5] structure is designed with the help of computer simulation studies with CFD ACE (CFD Research and Consulting Software GmbH, Berlin, Germany) where the geometry of the structure was optimized to obtain stable and adjustable coaxial sheathing (Figure 5). The structure was realized in silicon by reactive ion etching. Visual inspection of fluorescent beads passing through the structure and quantitative measurements of bead speeds clearly demonstrated the success of generating coaxially sheathed flows with this structure (Figure 6). The diameter of the core stream can easily be changed by altering the ratio of sample to buffer flow speeds.

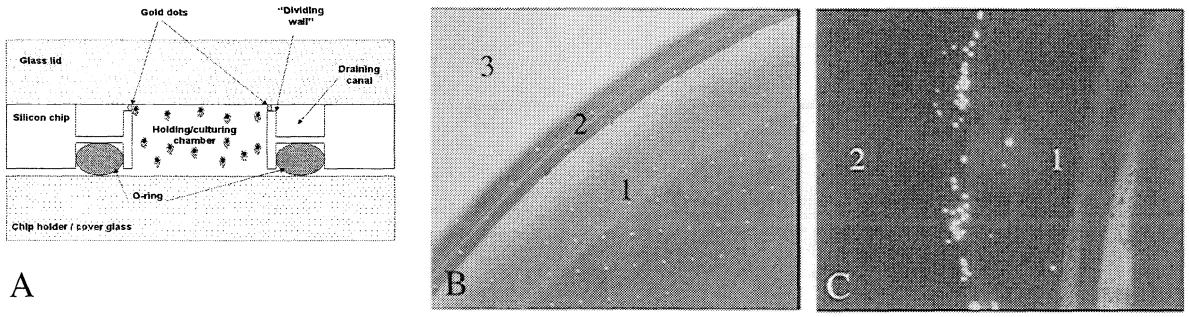

Figure 7: A) Schematic view of the holding/culturing chamber. B) Microsieve that separates the holding/culturing chamber from the draining channel. 1. holding/culturing chamber, 2. separation wall where gold bumps prevent complete sealing of the silicon to the glass cover plate, and 3. draining channel for removal of excess liquid or addition of nutrients for cell culture. C) Confinement of fluorescent latex beads in the holding/culturing chamber. 10 μm beads stay are concentrated along the rim of the chamber whereas 4 μm beads partly pass into the sieve structure. 1. holding/culturing chamber, 2. draining channel.

Probably the most challenging task in rare event sorting is the recovery of sorted cells. Collection of sorted beads or cells by pumping cells off chip through teflon or glass capillary tubing introduces large dead volumes and increased surface area, resulting in significant loss of beads or cells. Certainly when aiming at fetal cell sorting for prenatal diagnostics the loss of fetal cells is not acceptable and it is apparent that sorted cells must be stored on chip until the end of the sorting procedure. This realization prompted the introduction of a holding/culturing chamber. The chamber lies at the end of the collection outlet and is connected to a draining channel by a microsieve. The draining channel allows dissepation of excess fluid from the chamber whereas the microsieve prevents cells from leaving the structure. Alternatively, cells confined in the holding chamber can be cultured after the sorting process by feeding fresh culture medium through the draining channel. The microsieve was realized by locally preventing anodic bonding of the Pyrex glass plate to the silicon microstructure by introducing an array of 500 nm high gold bumps (Figure 7). The holding/culture chamber is fitted with a removable cover glass from the back by means of o-rings to allow access to the chamber after finishing the sorting procedure.

The components that were developed to improve rare event cell sorting were integrated in a single microstructure (Figure 8). The performance of this second generation cell sorters is under investigation.

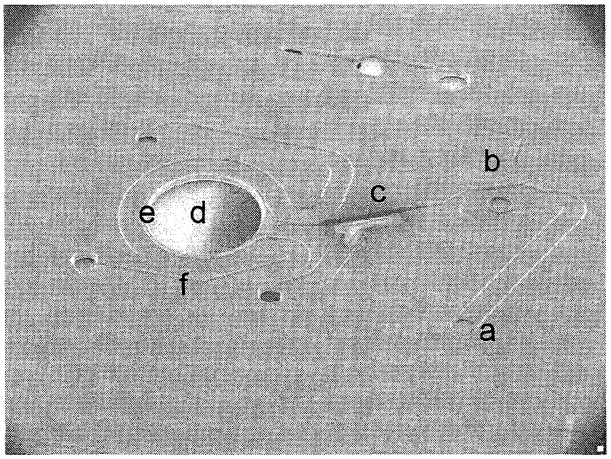

Figure 8: Micro cell sorter. a) buffer inlet, b) "chimney" structure for single step coaxial flow, c) glass cuvette for optical detection consisting of a glass membrane on the bottom of the channel and the Pyrex glass lid on top, d) holding/culturing chamber, e) microsieve, and f) draining channel.

2.3. Integrated Optics for Cell Analysis

Optical detection has proven to be a highly reliable, extremely sensitive and versatile tool, which is capable of fulfilling the high demands prompted by cell analysis[6]. Demands from telecommunication have fuelled the development of microfabricated optical components like waveguides and diffractive optics[7]. This development of integrated optics provides an alternative to expensive bulk optics requiring precise alignment. Once light is confined in waveguides it can be manipulated with splitters, couplers, and gratings. Although the specifications of the components for telecommuncation are very different from those for cell analysis applications, these components can be adapted for use in detection systems[8].

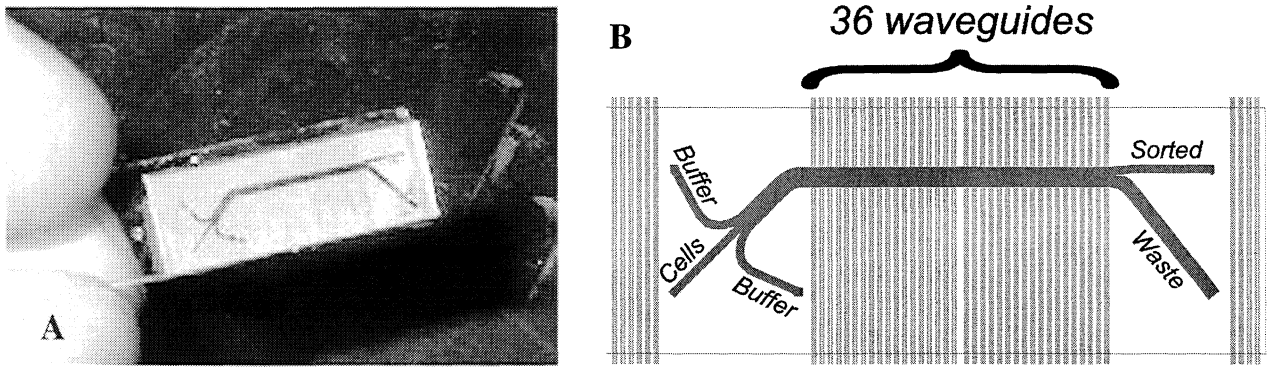

Figure 9: A) Photograph of microfluidic structure with integrated waveguides. B) Schematic depiction of the layout of microfluidic channels and waveguides.

The microfabrication, monolithic integration, and characterization of waveguides is described in detail by Kutter *et al*[9]. The first generation cytometer with integrated optical components used the same design as described in the beginning of section 2.2 (Figure 9). 36 waveguides with 4 μm high and 6 or 24 μm wide waveguide cores cross a 500 μm wide and 30 μm deep microfluidic channel. Cells are hydrodynamically focussed by sheathing with two flanking buffer streams.

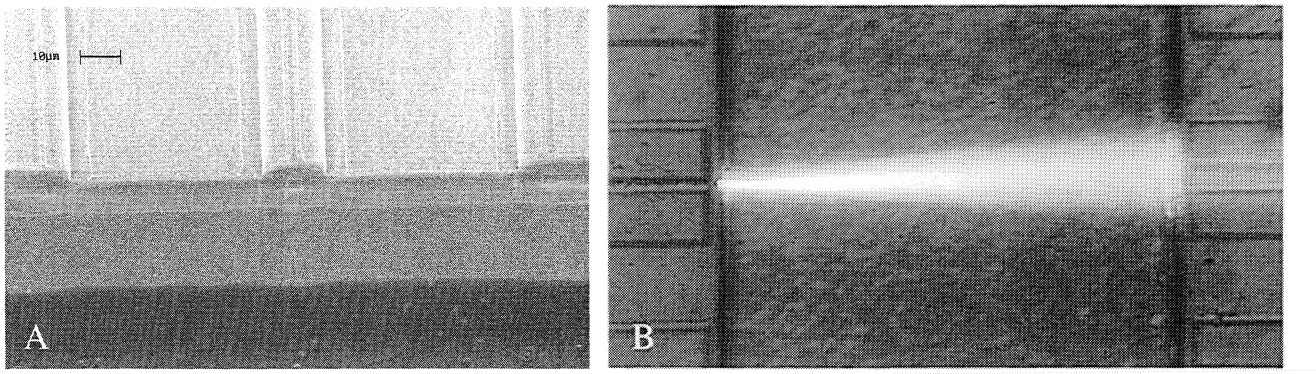

Figure 10: A) Scanning electron microscope image of waveguides monolithically integrated with fluidic micro channels. B) Light from an argon ion laser (488 nm) is coupled into a waveguide and is coupled from the waveguide into the 500 µm wide channel containing a solution of fluorescein that emits green fluorescent light.

Light from a laser is coupled into a waveguide that subsequently couples light into the channel (Figure 10) where it excites cells or particles marked with fluorochromes. Emission and scattered light is collected perpendicular to the channel through the Pyrex lid and registered with PMT's.

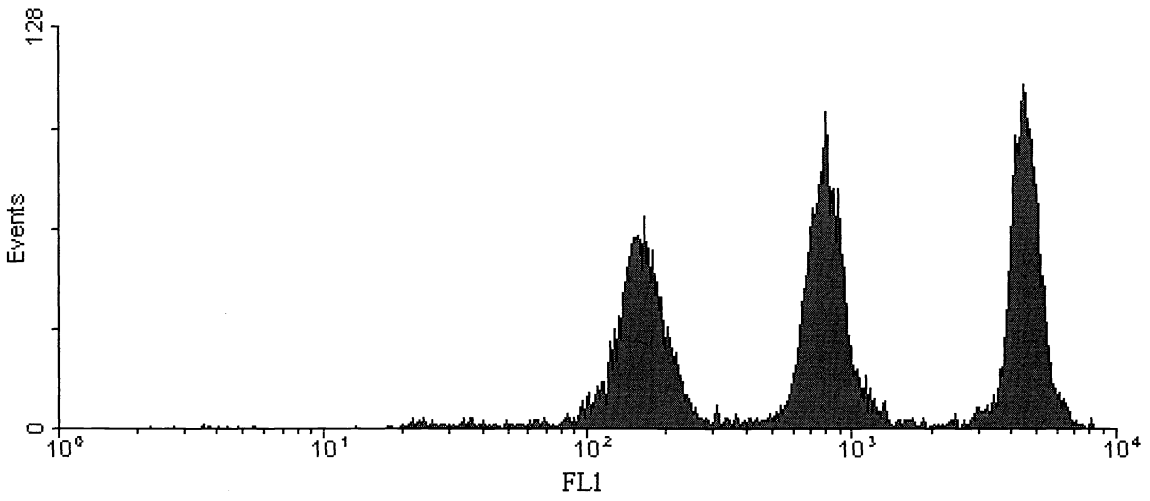

Figure 11: Identification of populations of particles with different fluorescent intensities in a microfluidics structure containing integrated waveguides.

To test the feasibility of using waveguides, particles with varying fluoresence intensities are guided through the structure. Fluorescence measurements are triggered by the scattered light and sorted according to fluorescence intensities (Figure 11). These initial results clearly demonstrate the potential of integrated optics for optical detection in microstructures.

We have yet to take full advantage of manipulation of light contained in waveguides. Different geometries of waveguides and microchannels (Figure 12A) for delivery and collection of light, filtering of specific wavelengths by means of Bragg gratings, and integration of photodiodes for conversion of optical signal to electronic signal (Figure 12B) are under investigation.

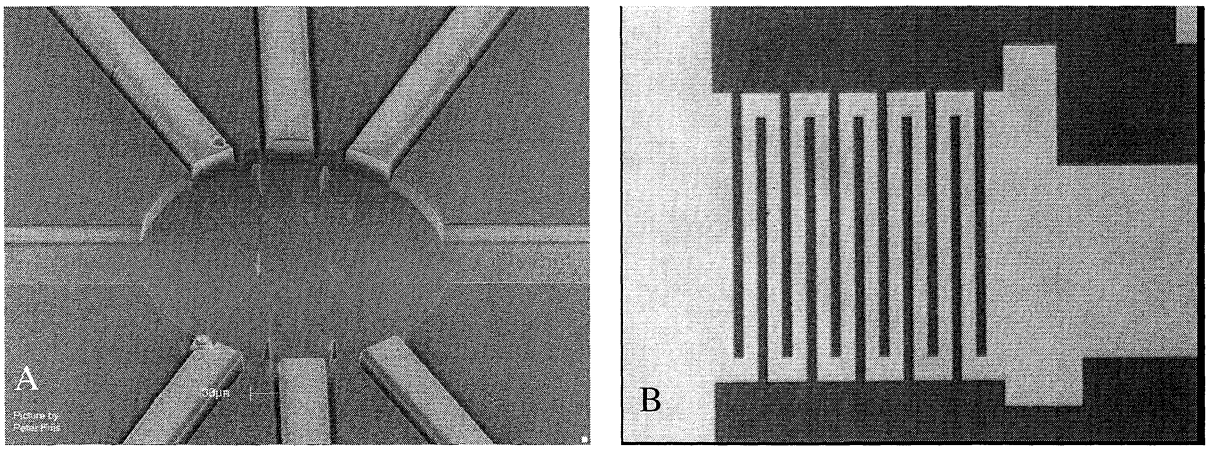

Figure 12: A) Using intergrated waveguides complex arrangements of waveguides and microchannels are possible. In this case 6 waveguides point to the center of a chamber that has a fluidic inlet and outlet. B) Integrated photodiode allowing the conversion of light contained in a waveguides to an electronic signal.

3. CONCLUSIONS

Electronic as well as optical detection of cells has been realized in hybrid devices where micro fabricated elements are interfaced to conventional instruments like pumps, light sources, detectors, and electronics. These hybrid devices have been succesfully used to analyze and sort particles and cells based on various parameters, e.g. cell size, cell number, and fluorescence intensity. The first level of functional integration on chip has been achieved. Future research will focus on further functional integration as to arrive at devices that are truly unique and cannot be realized with conventional techniques. The integration of cell analysis tools with DNA analysis tools like capillary electrophoresis and DNA microarrays are underway.

The strategy of miniaturization and functional integration that has been so succesfully used in the electronics industry is now starting to be applied to chemistry and life sciences. The potential benefits of this strategy are so large that it will undoubtetly result in a revolution in chemical, biochemical and cell analysis that is reminiscent of the development of computers. The initial results that have been shown in this field, which is effectively only 10 years old, confirm this notion. Many fundamental problems still need to be addressed to allow for the routine application of MST to chemistry and life sciences, the most pertinent being interconnection and packaging of μTAS. Answers to these two factors will determine the ultimate commercial success of μTAS. The need for a paradigm shift in analysis to satisfy the need of future research and industries is however so large that solutions to these problems will be found.

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