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Rare Cell Separation and Analysis by Magnetic Sorting

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Summary

The separation and or isolation of rare cells using magnetic forces is commonly used and growing in use ranging from simple sample prep for further studies to a FDA approved, clinical diagnostic test. This grown is the result of both the demand to obtain homogeneous rare cells for molecular analysis and the dramatic increases in the power of permanent magnets that even allow the separation of some unlabeled cells based on intrinsic magnetic moments, such as malaria parasite-infected red blood cells.

The continual development of the molecular understanding of biology, and especially human pathology, continues to push the limits of technology. A commonly discussed term is “personalized medicine” where a physician customizes a patient’s treatment based upon that individual’s specific molecular phenotype. However, such an approach increasingly requires the separation and analysis of cells from each patient. In most cases, a patient’s sample is difficult to analyze because the cells of interest are often rare in a complex mixture of heterogeneous cells.

The recognition of the need for simple and robust technology, in what is commonly called “point of care” systems in clinical application, has resulted in a large number of publications in recent years on various approaches to achieve the goals of personalized medicine. It is not possible to review all these various approaches in this article; consequently, we will focus on technologies utilizing magnetic forces to accomplish separation, with a brief mention of technologies based on microfluidics. In practical, clinical laboratory applications one is often confronted with a problem of weak molecular signal associated with the rare cell of interest as compared to that of normal tissue, such as blood cells, requiring a necessary step to reduce the molecular “noise” from normal cells. Separation is achieved based on the differences in cell physical properties, such as density and size, or more specific biochemical properties, such as surface antigen expression.[1] Advanced sorting techniques focus on separations based on surface antigen expression, such as Fluorescent-activated Cell Sorting (FACS)[2, 3] and magnetic cell separation[4]. They have rapidly evolved over the recent years driven by the ever increasing clinical demands and are now capable of separating cells with high selectivity and recovery.

As with the challenge of discussing the various cell separation technologies, it is equally challenging to characterize and discuss the various types of rare cells that are of scientific, clinical, public health, safety, and commercial interest. Examples include exogenous pathogenic bacteria in food, pathogenic micro-organisms used as weapons, and micro-

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organisms in animal fluids and tissue. Of even more challenge is the isolation of endogenous, rare cells in animal and human fluids e.g. hematopoietic stem cells (HSCs) in blood, circulating endothelial cells (CECs) in blood, and circulating tumor cells (CTCs). Given the large interest in the isolation of rare cells in human blood, we will limit this article to those technologies that are typically used on human blood and are in the most widespread laboratory use, based on optical and magnetic cell sorting methods

Modern tools such as FACS and magnetic cell sorting rely on the interaction between cell surface antigens and antibodies conjugated to fluorochromes or magnetic particles, and therefore, these techniques can be very specific to targeted cells. Since virtually all untreated biological materials are diamagnetic or only weakly magnetic, magnetic cell separation can be highly specific and is applied to cell mixtures as complex as whole blood. The current effort in magnetic separation focuses on miniaturization,[5–9] in flowing or static media, by either selective immobilization and deposition[7] or by magnetic deflection.[9–12] However, the magnetically deposited cells may be difficult to recover compared to the magnetic deflection-based systems, and therefore require direct interrogation *in situ* by optical means,[13] or electrochemically. Both permanent magnets[5] and electromagnets[7, 14] have been utilized as the source of magnetic gradients. On a general note, the choice of the separation method depends on the type of cells and its particular application, and therefore it is best presented together with the problem it aims to solve. The featured applications include difficult problem of CTC enumeration from blood samples obtained from patients with metastatic cancer disease.

Rare circulating cells as a cancer biomarker

The genesis of metastases in cancer is generally accepted to be the result of cells that dissociate from primary tumors and obtain access to circulation, either directly into blood vessels or after transit in lymphatic channels. With confirmation of cancer cells in the circulation over 50 years ago, one of the challenges has been developing technology with sufficient sensitivity and specificity to reliably examine the role of these circulating tumor cells (CTC) in cancer biology, the metastatic cascade, and as biomarkers for early detection.[15]

Accurate enumeration of CTC may provide insight into the processes of tumor growth and regression, metastasis, and disease progression and may allow discovery of the linkage between CTC, circulating endothelial cells (CECs), and clinical status.[16] From a research perspective, genotyping and phenotyping of CTC and CEC should provide insight into tumor biology, the metastatic process and permit direct exploration of targeted treatment strategies.[17]

It is generally assumed that metastases depends on sequential events, including a switch to the angiogenic phenotype and proliferation of blood vessels at least partially triggered by angiogenic proteins secreted by tumor cells. Thus, the identification of not only CTC but the addition of CECs, as a manifestation of the active but disorganized process of angiogenesis, could lead to important new biological and clinical insights. Increased numbers of CEC occur also in patients with myocardial infarction, infectious vasculitis, kidney transplant rejection, and cancer.

There is a growing body of literature which suggests that epithelial cancer undergoes a process called epithelial-mesenchymal transition (EMT) in which the tumor cells undergo a loss of polarity, lose cell-cell junctions, and acquire a mesenchymal phenotype, which promotes the escape of the cancer cells from the primary site and leads to the development of metastases.

There are a number of important similarities between organogenesis and tumors. Both of them have a heterogeneous population of cells of different phenotype and proliferation capabilities. Tumorigenic cancer cells self-renew and differentiate through processes similar to normal stem cells. Cancer stem cells (CSCs) have also been thought of as causing drug resistance and relapse of cancers. Putative CSCs have been identified in various solid cancers including breast, prostate, colon, brain, pancreatic, and head and neck cancer. It has been shown that CD44 expression is related to tumorigenicity. There is also evidence of the presence of CD133+ stem cells in breast cancer as well as in brain and colon cancer. A number of other cell surface markers have been suggested to identify these CSC in squamous cell carcinoma including: CD44+/CD24^{low}/lin^{-} CD133, ALDH1, and CD29. Recent progress in understanding the role of CTC in cancer was made possible by rapid development of cell separation technology, in particular the magnetic cell separation techniques.

CTC enumeration and analysis by optical methods

An automated process of CTC analysis in a model system of a breast carcinoma cell line (BT-20) spiked into peripheral blood mononuclear cells (PBMCs) using a specialized, multilaser flow cytometer and an automated gating algorithm has been described as early as 1995[18]. The limit of detection was 1 CTC in 10^7 PBMCs; however, it was pointed out that the process, if applied to whole blood, would require 200 mL blood sample, clearly an unrealistic sampling volume (and an unrealistically long processing time). A purging of the sample of erythrocytes and PBMCs prior to analysis was suggested and numerous current processes use one or more pre-enrichment steps.

Rapid improvement in optical detection methods opened the possibility of using laser scanning cytometry for CTC detection directly on blood smears on glass slides[19] A specialized system termed fiber-optic array scanning technology (FAST) has been tested on a model of metastatic colorectal tumor (HT29) spiked into whole blood from volunteer donors. The erythrocytes were removed by lysis and the remaining PBMC fraction was deposited on glass slides and stained for pan cytokeratin and cell nucleus markers. The combination of FAST screening followed by re-scanning of “hits” with a more conventional automated digital microscopy (ADM) resulted in average specificity of 1.5×10^{-5} and an average sensitivity of 98% at a scanning speed of 100 million PBMCs per hour (equivalent to approximately 5 mL whole blood per hour, excluding sample prep time). Interestingly, the authors applied their technology to check for false negative results of CTC detection by a positive immunomagnetic CTC separation method based on expression of the epithelial cell adhesion molecular (Ep-CAM) marker (using MACS microbeads and MiniMACS columns from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). They reported two potential issues with the positive CTC enrichment when compared to FAST+ADM scan: 1) the positive immunomagnetic separation leads up to 50% CTC losses in the process and 2) the positive immunomagnetic separation is highly sensitive to Ep-CAM marker down-regulation. The Ep-CAM and HER2/neu marker down-regulation has been observed in clinical studies of metastatic cancers[19].

Magnetic CTC separation

Magnetic cell separation technologies, including those reported to be used to isolate CTC, typically require a specimen cell suspension to be incubated with magnetic beads coated with antibodies directed against epithelial or endothelial cells; the epithelial cells are then isolated using a powerful magnet. By combining a sample preparation platform that utilizes immunomagnetic enrichment and immunofluorescent labeling, Veridex LLC (a Johnson & Johnson Company) sells a system, CellSearch™, with the requisite sensitivity and

specificity to detect rare cells in a sample of whole blood (both based on early development work by Immunicon Corp.). The assay has FDA approval for detection of CTC in patients with metastatic breast cancer, metastatic colorectal cancer, and metastatic prostate cancer. Determination of CTC counts are made by trained operators with periodic independent validation by Veridex of results. The strict, quantitative criteria of the CTC definition and a highly automated CTC separation and analysis process that yields to validation generated considerable interest and has been successfully tested in multi-center trials. The technology enabled the first, evidence-based approach to prognosis of overall survival and disease-free survival following therapy of metastatic cancers based on CTC enumeration and comparison with a certain critical "cut-off" CTC concentration values in blood.[20] CellSearch technology relies on nanotechnology as it is built around functionalized magnetic nanoparticles (the term introduced by Immunicon Corp. is a "bioferrofluid"). The commercialization of this technology provided a benchmark for new, emerging technologies for CTC detection where an increased dynamic range of the CTC number and molecular composition of the reported "CTC" are the stated goals. It is expected that this competition between an existing commercial instrument and emerging technologies will provide additional important clinical information that goes beyond a simple "yes-or-no" answer.[21, 22]

Negative cell separation by depletion of unwanted cells is a way of enrichment of cells of poorly understood phenotype (such as CTCs with a metastatic or a "cancer stem cell" potential) and cells "untouched" by the labeling ligands. Both attributes are highly desirable for research on CTC biomarkers and specialized magnetic separation instrumentation and reagents are available commercially (albeit for less demanding applications than CTC enumeration in whole blood). We have recently developed a negative CTC enrichment strategy that relies on a combination of viscous flow and the magnetic force that facilitates recovery of the unlabeled cells (CTCs) from whole blood samples obtained from cancer patients.[23–25] The method is based on separations in axial flow in long cylinders placed in quadrupole magnets and combines advantages of using viscous flow stress and the magnetic field force to achieve high throughput and high enrichment rate (by as much as 10,000 fold)[23] It consists of removal of normal blood cells from the patient blood sample by erythrocyte lysis followed by pan-leukocyte marker (CD45) antibody labeling and depletion by magnetic separation. The remaining cells, if any, are treated as putative CTCs and are available for analysis for known CTC markers, described above, using immunocytochemistry and molecular biology (RT-PCR) methods.

The flow channels and the permanent magnet assembly used in this negative CTC enrichment technology is shown in Figure 1. Once an enriched cell sample is obtained from the magnetic separation step, it is used for RT-PCR analysis and for immunocytochemistry (ICC) analysis. Immunocytochemistry analysis typically consists of creating a cytospin on the enriched cell sample from the magnetic separation step and labeling this slide with DAPI to stain cell nuclei and a pan-cytokeratin antibody conjugated to FITC. The CTC must meet the following criteria: 1) be double positive for FITC and DAPI, 2) the cell must have an intact membrane, and 3) the cell must have a high nuclear-to-cytoplasmic ratio. An example of multi-marker analysis using confocal microscopy of CTC cell preparations obtained using negative CTC isolation is illustrated in Figure 2.

Magnetic separation of other types of rare cells

Transplantation of tissue progenitor cells has become an accepted clinical treatment modality and is an active area of research for applications to regenerative medicine. The current, FDA approved clinical applications include bone marrow transplantation and hematopoietic stem cell (HSC) transplantation from consenting donors (the so-called adult HSCs) to counteract the inevitable side-effects of aggressive chemo- and radio- therapy of

cancer, suppressing the hematopoietic (blood forming) activity in the patient. This includes autologous transplantation in which patients serves as his/her own donor, or an allogeneic transplantation in which an HLA-matched related family member, or a partially HLA-mismatched, unrelated person serves as a donor. The challenge is that the hematopoietic progenitor cells are rare. The frequency of progenitor cells in the bone marrow is about 1 in 100,000 nucleated cells. The peripheral blood of a healthy donor contains even fewer progenitor cells (which, however, can be significantly increased by mobilizing cells from the marrow by administration of recombinant human growth factors, G- or GM-CSF). Therefore, the stem cell isolation techniques have become an early, critical component of the curative strategy. Apart from its significance in the therapeutic reconstitution of hematopoiesis in cancer patients, stem cell separation is important in clinical research on the identification of hematopoietic long-term colony-initiating cells (LT-CIC), capable of sustained engraftment in the recipient, such as cells expressing CD34 surface molecule. Yet another important application of cell separation is in the generation of a large number of tumor-reactive T lymphocytes for adoptive immunotherapy of cancer. The therapy relies on isolation of tumor-reactive lymphocytes from patients with malignancies, *ex vivo* activation and expansion of such lymphocytes, and their reinjection to the patient where they home in and eradicate the tumor.

Considerable effort has been spent on developing methods for manipulating, or "engineering", the graft for a successful hematopoietic cell repopulation,[26] and extensive reviews of these methods have been published. These include immunomagnetic separation, the antibody and complement-mediated purging of tumor cells, T cell depletion by immunotoxins, avidin-biotin affinity columns for positive progenitor cell selection, and counter-current elutriation. A highly successful open-gradient line of separators was designed around micrometer-sized Dynabead magnetic particles for clinical cell separation applications (from Invitrogen Inc.).[27, 28] Due to their relatively large sizes (4.5 μm or 1 μm) the preferred applications of the Dynabeads is to negative cell selection, where the cells targeted by the magnetic label are discarded, and only the unlabeled cells are used. On the other hand, the use of colloidal magnetic particles as magnetic labeling reagents offers advantages in forming stable suspensions and fast reaction kinetics, similar to immunofluorescence labels.[29] The small size of the particles, in the range of tens to a hundred nanometers, comes with the cost of low magnetic moment, requiring high fields and gradients, and therefore the use of specialized, magnetic affinity-type columns, typically obtained by soft steel alloy inserts inside the cell suspension container. A particularly successful design has been developed and commercialized by Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, around the magnetic colloid of trade name MACS MicroBead™.[4] The company offers a full line of products, from small, laboratory use MiniMACS columns to large, automated separators currently undergoing clinical trials for hematopoietic stem cell enrichment for allogeneic stem cell transplantation, CliniMACS.[30] A second commercial design, based on a multipole magnetic field that is coaxial with the cylindrical shape of the laboratory tube and used in combination with magnetic dextran colloid, is available from StemCell Technologies, Inc., Vancouver, Canada.

Label-less magnetic separation of malaria parasite-infected erythrocytes

The gold standard for malaria diagnosis remains the microscopic examination of Giemsa or Wright's stained blood smears. The diagnosis is relatively inexpensive, gives a measure of parasitemia and an identification of malaria species, but is time consuming and requires a skilled microscopist. An experienced microscopist can obtain a detection limit of around 50 parasites/μL of blood smear, which is equivalent to 0.001% parasitemia. Many labs routinely achieve a sensitivity of only 500 parasites/μL (0.01%), however. In addition, between 200 and 300 microscope viewing fields must be examined before a negative report

is given. Therefore, development of other diagnostic methods is actively pursed. Diagnostic tests based on PCR amplification of target RNA sequences can be extremely sensitive, with detection levels of <5 parasites/μL (0.0001%) possible. However, the detection is not quantitative, and RNA may persist and be detected for 6 days following the elimination of parasites, and the technique cannot compete with blood smear microscopy on cost.

In an attempt to overcome some problems inherent to blood smear microscopy, a magnet-based approach was developed to concentrate malaria parasites and augment detection of malaria-infected erythrocytes by microscopy, Figure 3A. This system, malaria magnetic deposition microscopy (MDM), exploits the fact that *Plasmodium* species parasites produce a crystalline by-product, hemozoin, from heme liberated during hemoglobin digestion. Unlike previous systems requiring elution of cells from steel mesh[31, 32], MDM captures parasitized erythrocytes in a narrow magnetic field and deposits them directly onto a small region of a polyester slide, Figure 3B, which is then immediately ready for fixation, staining and viewing, Figure 4. By concentrating parasites, MDM increases the sensitivity of diagnosis and decreases the time it takes to read the slide, of all four human malaria parasite species, including efficient capture of *P. falciparum* gametocytes.

Other technologies for improved rare cell capture

The promise of the cell-based diagnostics and cellular therapeutics has spurred development of new technologies in recent years and renewed interest in other technologies proposed earlier for rare cell separation. The limited space allows only for a passing mention of selected techniques and the interested reader will find additional information in the literature references. In particular, separation of cells by immunoaffinity capture in thin flow channels offers advantages of simplicity of operation and sample preparation and has been investigated for a number of years.[33] The application of the Micro-Electromechanical System (MEMS) technology to a microfluidic design enabled fabrication of microchannels with highly developed internal surface area using microposts and has been shown to allow for an efficient epithelial cell adhesion molecule (Ep-CAM) positive cell capture and detection from whole blood.[34] The flexibility and precision of the MEMS microchannel fabrication process has engendered interest in further exploring the technology to CTC detection applications The ability to selectively capture CTC from whole blood in a small area suitable for optical analysis currently limits the use of the MEMS technology to positive CTC isolation of a defined CTC immunophenotype (epithelial cells) or a defined CTC physical properties (large size). The idea of isolating the CTC from whole blood by filtration is based on an early observation that the tumor cell diameter tends to be larger than that of the normal blood cells (15 μm vs. not greater than 10 μm).[35] The early efforts led to setting up in the 1960's an NCI Circulating Cancer Cell Coordinating Committee to review the evidence for circulating cancer cells; however, the results were inconclusive, seemingly in part because of lack of quantitative criteria for the "circulating cancer cell".[36] The idea was revived by a publication describing the combination of CTC filtration and a molecular analysis of the filtered cell lysate that led to a commercial venture in France. It continues to be evaluated in a microfilter format[37] by taking advantage of improved filtering capabilities of microfluidics channels.[38] Another distinguishing physical property of the CTC is its mean dielectric constant related to highly developed endoplasmic reticulum, intracellular lipid bilayer content, and structural organization, which is the basis of the investigations on dielectrophoretic separation of CTCs.[39]

Recent advances, and future directions in cell separation techniques

As already mentioned above, the current efforts are mostly focused on adapting microfabrication technology for building cell sorting devices, and on a search for exploitable

cell sorting mechanisms.[40] These include miniaturization and modification of the optical detection methods and mechanical actuation methods adapted from larger instruments, complemented by investigations of other mechanisms that are important only at microscale, such as dielectrophoresis, and optical trapping.[41] Microfluidic systems are actively pursued typically as an enabling technology for these basic separation approaches targeting rare cell or for single-cell analysis.[42] In some cases, magnetic separation methods are applied in both these microfluidic and macro scale system, since magnetic separation can span the range of macro- to micro-separations, depending on the magnetic field, its gradient, and the size of the separand. Magnetic separation is also very effective in application to cell separation in microchannels because of the possibility of producing very high field gradients on the microscale.[43] Another appealing quality of magnetic forces is that they can provide both sensing and actuation functions, thus significantly simplifying cell sorting mechanics.[44] Magnetic cell separation is an active area of research and commercial applications because of these qualities spoken of above and the already well developed technique of magnetic cell tagging using monoclonal antibodies conjugated to magnetic nanoparticles.[8, 10, 29, 45] This magnetic nanoparticle tagging is a obvious extension of the antibody-fluorescent conjugate technology which is fundamental to flow cytometry and fluorescent microscopy.

At the most fundamental level, cell separation (beyond complete removal of all cells) requires a method to selected for the targeted cell, and a method to physically move (and or bind) this targeted cell. While there are many ways to identify a cell, and these techniques continue to be discovered and developed, the actual movement and physical separation of the targeted cell is limited by the exploitation of physical differences in the targeted cells from the contaminating cells. Such differences include: size, density, intrinsic dielectric or magnetic properties, or physical differences that are imparted onto the cell through some type of affinity binding of molecules and or particles. Consequently, for the foreseeable future, the development of separation technology for rare cells will most probably involve the refining of one of these known physical separation techniques.

For example, further refinement of magnetic separation systems can involve (but is not necessarily limited to) the orthogonal superposition of convective and magnetic transport which does not require sensors or actuators. The selectivity of such a separation is then based on the specificity of the targeting antibody-magnetic nanoparticle conjugate. The throughput is determined by the volumetric flow rate of the cell suspension, but is still limited by physical limitation such as those introduced by the field-induced velocity which affects the necessary length of the separation region. However, such cell sorting is based on the cell surface marker expression, without the complicated fluidics, sensing and actuation mechanics inherent to current optical sorting methods.[46] There is already a large body of data on similar systems such as field-flow fractionation, split-flow thin (SPLITT) channel fractionation and free-flow cell electrophoresis that is used for experimental design and data analysis,[47, 48] especially based on a useful concept of “transport lamina”.[12] Other potential future separation technologies using established physical principles include further refinement of filtration and dielectric fields. It is hoped that with the increasing interest in the need to obtain pure, or nearly pure cell subpopulations, especially when the targeted cells is rare, that cleaver new physical cell separation principles can be discovered to further push the field forward.

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Biography

Dr. Maciej Zborowski, a biophysicist, is a staff member at the Lerner Research Institute of the Cleveland Clinic (CC) and Associate Professor of Molecular Medicine at CC Lerner College of Medicine of Case Western Reserve University. He specializes in the fundamentals and applied aspects of magnetism and biology since 1985. Dr. Jeffrey J Chalmers is a Professor in the Department of Chemical and Biomolecular Engineering at The Ohio State University and is director of the Analytical Cytometry Shared Resource at the Ohio State University Comprehensive Cancer Center. He has specialized on the interactions of hydrodynamic forces and cells and, in collaboration with Dr. Zborowski, has collaborated on the fundamentals and applied aspects of magnetism and biology since 1992.

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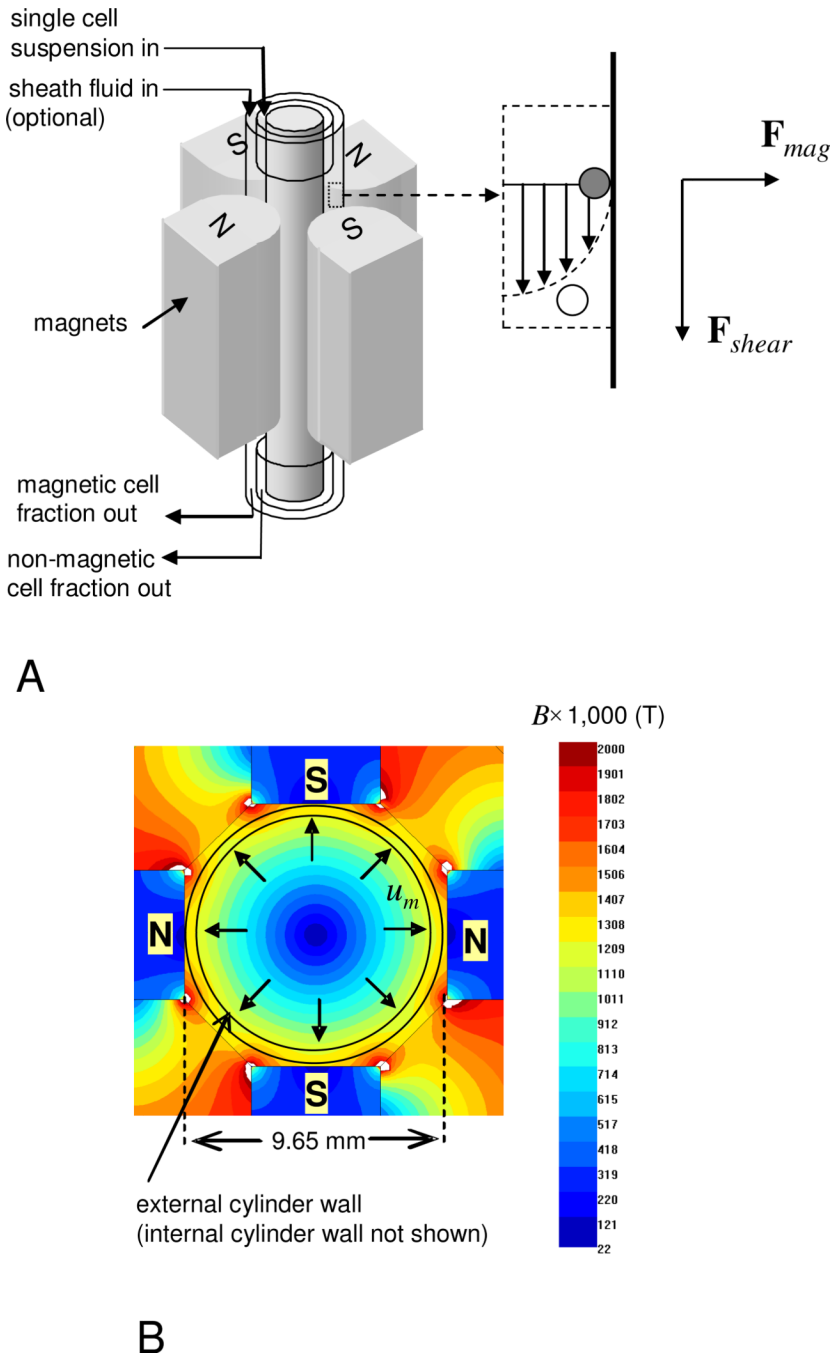

Fig. 1.

A) Diagram of the quadrupole magnetic flow sorter (QMS) system. The four pole pieces are arranged in N-S-N-S configuration and exert a radial force on the magnetically labeled cells (grey circle), removing them from suspension by deposition on the channel wall. The unlabeled CTCs (open circle) are collected as “non-magnetic fraction” downstream. Other flow configurations are also shown which can be used for added operational flexibility of the flow sorter by taking advantage of a “sheath fluid” and a continuous collection of the “magnetic fraction”. The magnetic, $\mathbf{F}_{mag}$, and fluid shear, $\mathbf{F}_{shear}$, forces are complex functions of the magnetization and quantity of the magnetic label attached to the cell, local field intensity and field gradient, fluid viscosity and the volumetric flow rate, and channel

dimensions. B) The radial direction of the magnetic force acts as a “magnetic centrifuge” by pushing the magnetically labeled cells against the cylinder wall, without affecting the unlabeled cells.

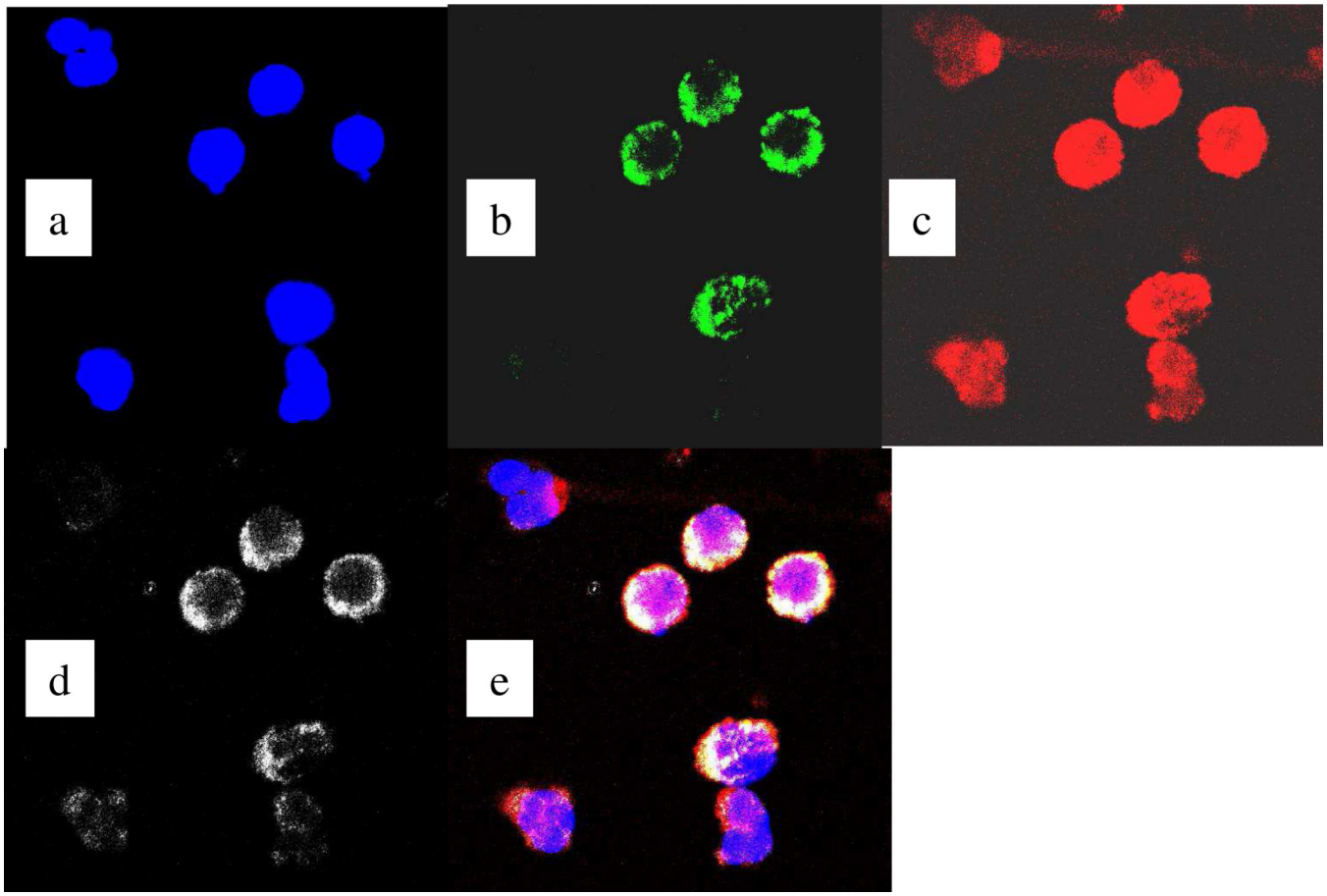

Fig. 2.

Immunocytochemical analysis of NIMS cytospin preparation shows putative CTCs that are also positive for vimentin and CD44 markers (panel **e**). Here **a** nuclei (DAPI), **b** cytokeratins (FITC), **c** CD44 (APC) and **d** vimentin (Alexafluor). Panel **e** is a composite of **a–d** images.

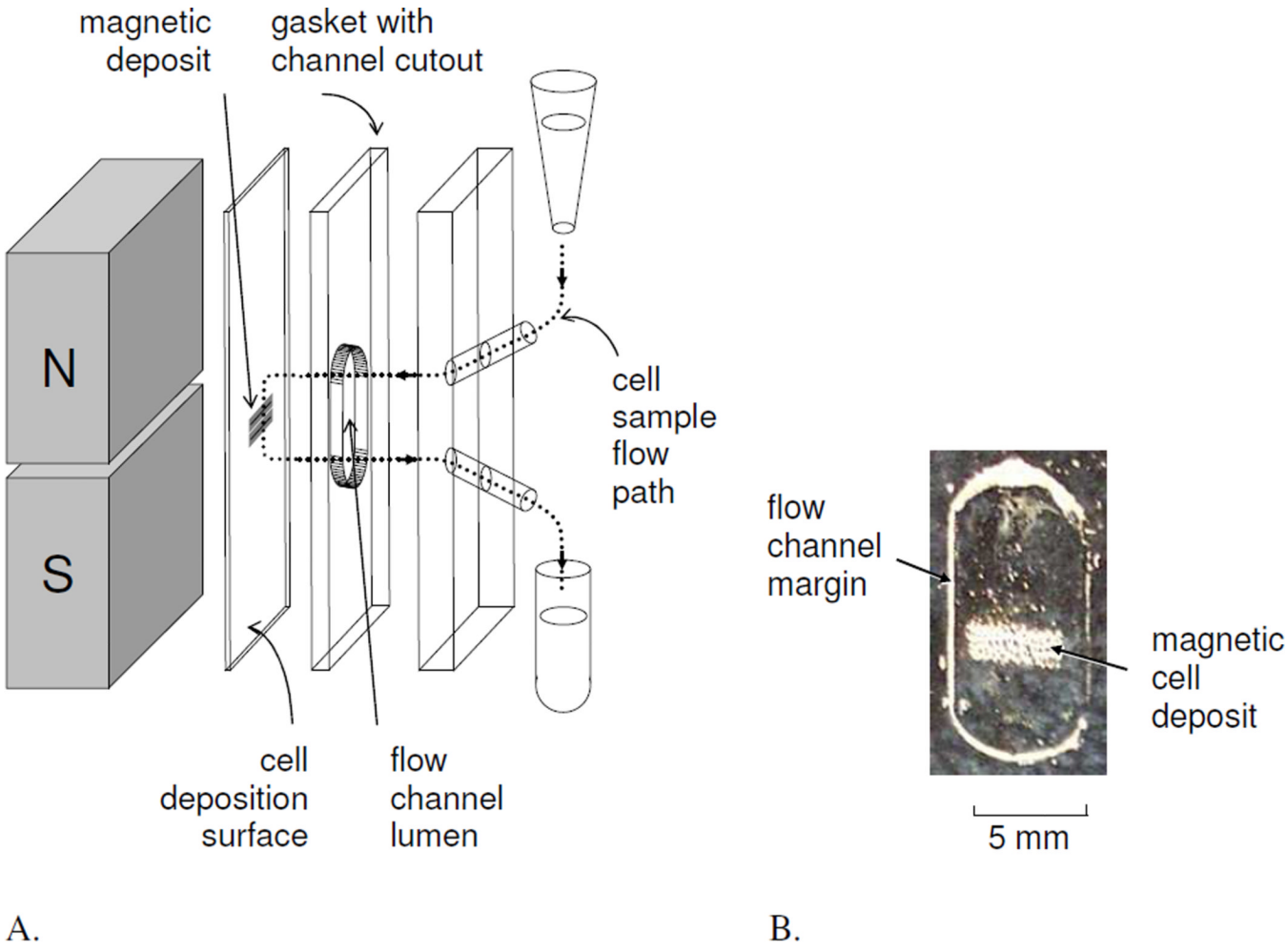

Fig. 3.

A. Components of the malaria MDM device and the sample flow path. Note the location of the expected magnetic cell depositband next to the magnet pole piece tips (drawing is not to scale). B) An un-aided eye appearance of the magnetic deposition, collected in the interpolar gap area (Panel A and B), from a *P. falciparum* parasitized blood sample.

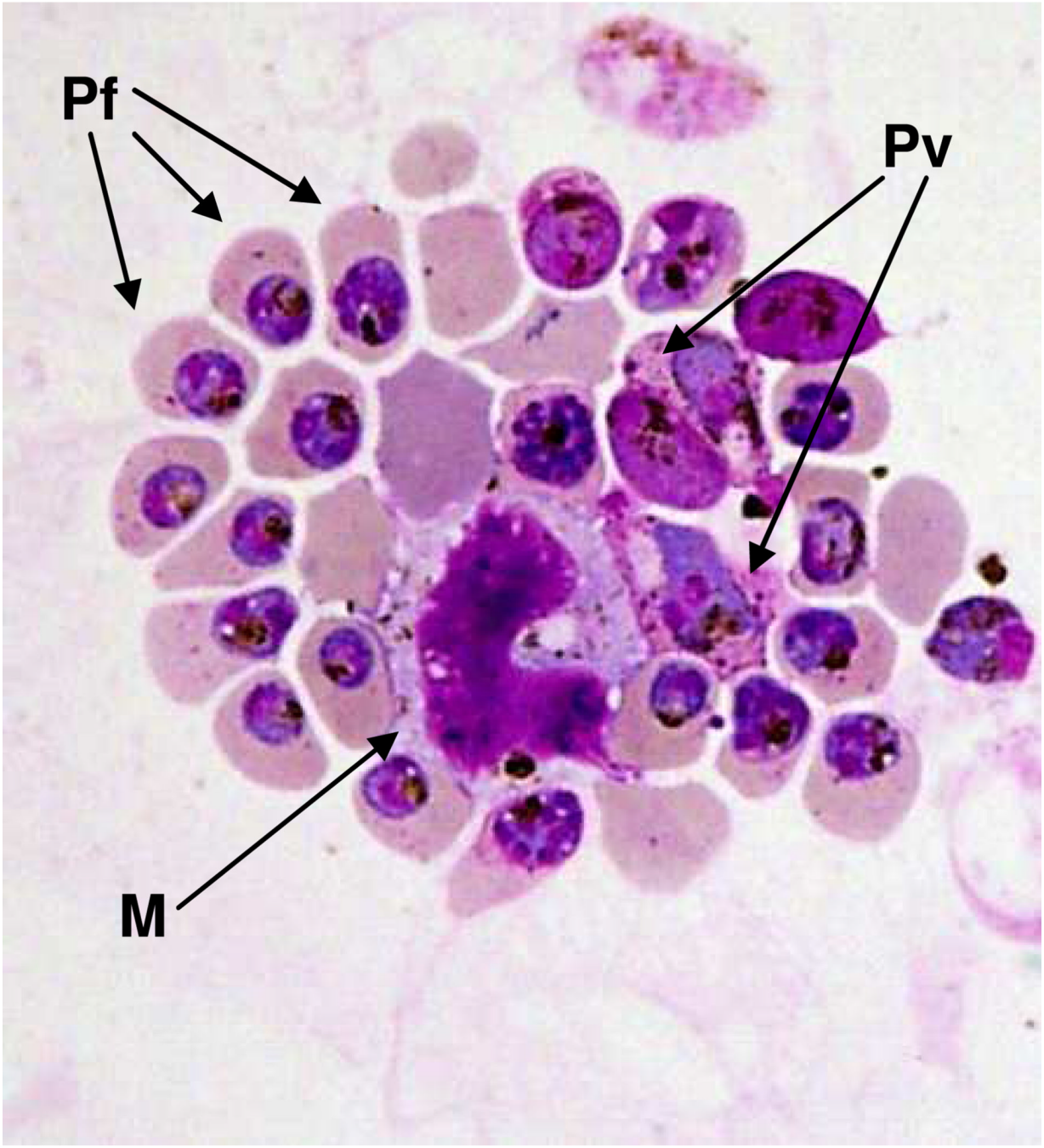

Fig. 4.

Magnetic deposition microscopy detection of *P. falciparum* and *P. vivax* from a mixed blood sample. Equal volumes of blood from *P. falciparum* (initial parasitemia of 2.7%) and *P. vivax* (initial parasitemia of 0.1%) infected monkeys were mixed and then subjected to MDM analysis. Giemsa stained slides show MDM concentration of *P. falciparum* (Pf), *P. vivax* (Pv) and macrophages (M) containing hemozoin.