# Application of an improved magnetic immunosorbent in an Ephesia chip designed for circulating tumor cell capture

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**Abbreviations: CTC**, circulating tumor cell; **EDC**, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; **EpCAM**, epithelial cell adhesion molecule; **IS,** immunosorbent; **PGMA**, polyglycidyl methacrylate; **S-NHS**, *N*-hydroxysulfosuccinimide sodium salt

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**Abstract**

In this study, we describe a particular step in developing of a microfluidic device for capture and detection of circulating tumor cells (CTCs) – specifically the preparation of an immunosorbent (IS) for implementation into the separation chip. We highlight some of the most important specifics connected with superparamegnetic microspheres for microfluidic purposes. Factors such as nonspecific adsorption on microfluidic channels, interactions with model cell lines, and tendency to aggregation were investigated. Poly(glycidyl methacrylate) microspheres with carboxyl groups were employed for this purpose. To address the aforementioned challenges, the microspheres were coated with hydrazide-PEG-hydrazide, and subsequently anti-EpCAM (epithelial cell adhesion molecule) antibody was immobilized. The prepared anti-EpCAM IS was pretested using model cell lines with differing EpCAM density (MCF7, SKBR3, A549 and Raji) in a batchwise arrangement. Finally, the entire system was implemented and studied in an Ephesia chip and an evaluation was performed by the MCF7 cell line.

## 1 Introduction

Among other important topics, cancer research is focused today on circulating tumor cells (CTCs) because this biomarker has in recent years confirmed its high potential in therapeutic response monitoring, diagnostics, and as a prognostics tool [1] for various types of metastatic cancer, such as breast [2-6], lung [7-9], prostate [10, 11], gastric [12, 13], colon [12, 14] and bladder cancer [15-17]. Underscoring the importance of this work is the fact that, according to the World Health Organization, cancer is one of the leading causes of death worldwide.

Therefore, new technologies for CTCs capture, characterization and post-capture analysis on viable cells are being intensively developed, and especially in the field of microfluidics in keeping with the trend toward miniaturization. Most of the microfluidic devices are based on cell immunocapture strategy using antibodies immobilized on magnetic beads [18-20] or directly on the channel walls and/or obstacles within the channel [21, 22]. The antibodies utilized are usually specific to some particular CTCs' surface molecules. Most generally, this is epithelial cell adhesion molecule (EpCAM), but prostate-specific membrane antigen (PSMA) also has been proposed in the case of prostate cancer [23]. Another approach to microfluidic strategy works with physical characteristics of CTCs, such as size [24] or stiffness [25, 26], and with electrical properties [27-29]. Although interesting, those "label free" methods still lack of strong clinical studies to promote them [30]. Commercial products such as On-Q-ity's C5, Biocept's CEE™, and ApoCell's ApoStream™ [31], among others, have been developed for CTCs capture. To date, however, Veridex's CellSearch® is the only device that has been approved by the US Food and Drug Administration for CTC enumeration. This system uses anti-EpCAM antibody-coated magnetic nanoparticles for CTC capture which are then separated by magnetic field. The disadvantage of this assay is that it requires multiple processing steps, such as centrifugation, dilution, capture, and separation,

and this leaves the captured cells nonviable [32]. Nevertheless, and in spite of certain drawbacks, this system has already been used in many research or clinical studies and has enabled comparison of results from different research groups.

In our study, we worked with an Ephesia chip made of PDMS, which combines a microfluidic cell with immunomagnetic sorting [18, 19]. This recently developed technology involves a diamond-tree-shaped microfluidic system wherein multiple inlet channels bring the sample into a microfluidic flow cell where an anti-EpCAM micropillar array made of self-assembled microspheres is formed using an external magnetic field. As flow resistance of micropillars as well as CTC capture efficiency depend on the physicochemical properties of the immunosorbent (IS), it is crucial to have the right selection of magnetic microspheres with appropriate characteristics (in particular magnetization), quality mAb clone, and method of their surface coating and biofunctionalization.

Superparamagnetic poly(glycidyl methacrylate) microspheres with carboxyl groups (PGMA-COOH), 4 μm in diameter, were used in this study because they fulfilled the basic requirements concerning size, material, surface groups, and magnetic characteristics. Nevertheless, some nonspecific adsorption onto microfluidic PDMS channels and inserted cells was observed. Thus, surface coating was applied using PEG in form heterobifunctional hydrazide-PEG-hydrazide. The level of nonspecific adsorption of microspheres onto the cells and/or PDMS channels before and after PEG-coating and/or anti-EpCAM antibody immobilization was investigated. Capture efficiency of the improved anti-EpCAM IS was tested initially on cell lines with different EpCAM densities (MCF7, SKBR3, A549 and Raji) in a batchwise arrangement. Final evaluation in a microfluidic Ephesia chip was demonstrated using the MCF7 cell line, a model system for CTC. All obtained findings are thoroughly discussed herein.

## 2 Materials and methods

### 2.1 Materials

*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide sodium salt (S-NHS), MES, sodium periodate, ethylene glycol, BSA, nonspecific human IgG isolated from serum, Hoechst 33258, PBS, and other chemicals used for buffer preparations were from Sigma-Aldrich (St. Louis, MO, USA). PEG (amino-PEG-carboxyl, hydrazide-PEG-hydrazide) 3400 kDa was from Laysan Bio (Arab, AL, US). Dynabeads® Epithelial Enrich and Dynabeads® M-280 Tosylactivated were purchased from Life Technologies (Carlsbad, CA, USA). Mouse monoclonal anti-EpCAM antibody IgG1 (HEA 125) was provided by PROGEN Biotechnik (Heidelberg, Germany). Microspin™ G-25 columns were from GE Healthcare (Buckinghamshire, UK). Laemmli and tricine sample buffers were from Bio-Rad (Hercules, CA, USA). Human breast adenocarcinoma MCF7, SKBR3 and Raji cell lines were purchased from Health Protection Agency Culture Collections (Salisbury, United Kingdom), and carcinomic human alveolar basal epithelial cell line A549 was from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell cultivation reagents were from Life Technologies (Carlsbad, CA, USA). Ultrapure Q-water ultrafiltered on a Milli-Q Gradient A10 system (Millipore, Molsheim, France) was used throughout the work.

### 2.2 Cell cultivation

The MCF7, SKBR3 and A549 human cell lines expressing EpCAM (EpCAM+) were cultured in DMEM supplemented with aqueous penicillin/streptomycin (100 μg/mL) and 10% (v/v) fetal bovine serum at 37°C in a humidified 5% $CO_2$ atmosphere, and 0.01 mg/mL of insulin was added in the case of MCF7. RAJI cells (no EpCAM expression) used as a

negative control for cell capture were cultured in RPMI-GlutaMax supplemented with aqueous penicillin/streptomycin (100 µg/mL) and 10% (v/v) fetal bovine serum at 37°C in a humidified atmosphere with 5% $CO_2$. Cell suspensions were centrifuged at 300 *g* for 5 min and resuspended in PBS (pH 7.4). Cell numbers were determined using a CASY cell counter from Roche Innovatis AG (Reutlingen, Germany) and the desired concentration of cells was then obtained by dilution with PBS (pH 7.4). The cells were stained by Hoechst 33258 for 30 min at room temperature according to the supplier's instructions.

### 2.3 Microsphere preparation

The synthesis of monodisperse ammonolyzed macroporous poly(glycidyl methacrylate-*co*-[2-(methacryloyloxy)ethoxy]acetic acid-*co*-ethylene dimethacrylate) microspheres (PGMA) already has been thoroughly described in other papers [33-36]. Briefly, magnetic PGMA microspheres were prepared by poly(vinylpyrrolidone)-stabilized and 2,2-azobisisobutyronitrile-initiated dispersion polymerization of GMA in ethanol in the presence of colloidal magnetite treated with either perchloric acid or tetramethylammonium hydroxide. The polymerizations were run at 70°C for 16 h.

### 2.4 Microspheres surface modification by PEG

PEGylation was performed by the two-step carbodiimide coupling procedure in the presence of sulfo-NHS reagent [37]. The EDC (0.039 M) and sulfo-NHS (0.0057 M) were dissolved in 0.01 M MES buffer (pH 5) and were immediately added to 1 mg of washed microspheres. The activation of microspheres' surface groups ran at room temperature (RT) under stirring for 5 min. The supernatant was removed using a magnetic separator from Life Technologies (Carlsbad, CA, USA). Activated microspheres were resuspended in 0.01 M MES buffer (pH 5) containing 1.7 mg of PEG ($NH_2$-PEG-COOH or hydrazide-PEG-hydrazide), which is equivalent to a 2.5× molar excess of PEG compared to carboxyl content on the microspheres' surfaces. Immobilization proceeded overnight at 4°C under mild stirring. The PEGylated

microspheres were washed 7× with 1 mL of 0.1 M MES (pH 5). The microspheres were then incubated in 0.1 M MES (pH 5) with 1M NaCl for 15 min at room temperature under stirring to remove the unbound PEG, then 2× washed with 1 mL of 0.1 M MES (pH 5). A Nikon Eclipse 80i optical microscope (Nikon, Tokyo, Japan), equipped with Nikon Plan Fluor 10×, 20×, 40× and 60× objective lenses and a Nikon digital sight DS-MS camera, was used for final observation of their possible aggregation.

### 2.5 Immobilization of IgG onto PEGylated microspheres

The magnetic PGMA-COOH microspheres PEG-coated with hydrazide terminal group (1.0 mg) were 3× times washed with binding buffer (100 mM acetate buffer with 0.2 M NaCl; pH 4.6). A total of 25 μg of IgG was oxidized in 0.02 M sodium periodate for 30 min in darkness. The reaction was stopped by addition of 0.4 μL of ethylene glycol. The unreacted reagent was removed using G-25 microcolumns. The activated antibodies were added to the washed microspheres and incubation ran overnight at room temperature under stirring. The prepared IS was repeatedly washed with PBS (pH 7.4) and was stored in PBS with 0.05% (m/v) sodium azide and 0.1% (m/v) BSA at 4–8°C.

The immobilization efficiency of IgG attached to magnetic microspheres was evaluated by comparing the IgG solutions before and after the immobilization using SDS-PAGE (10% T, 3% C gel, 10 × 8.3 cm and 0.75 mm thick) according to Laemmli [38]. IgG samples were mixed with Laemmli sample buffer (1:1) and boiled at 100°C for 2 min. The SDS-PAGE was carried out in a Mini-PROTEAN Tetra electrophoresis cell (Bio-Rad; Hercules, CA, USA) and ran for 45 min at 180 V in Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS), with silver staining following [39].

### 2.6 EpCAM+ cells immunocapture in batchwise arrangement

The magnetic anti-EpCAM immunosorbents (0.5 mg per tube) were 3× washed with 1 mL of PBS (pH 7.4) containing 0.1% BSA using a magnetic separator. They were then mixed with $2 \times 10^6$ cells (optimized amount saturating the applied immunocapture system) and rolled on a rotator for 30 min at RT. In the next step, the microspheres with isolated cells were 5× washed with PBS (pH 7.4) containing 0.1% BSA to remove all unbound cells, and a new Eppendorf tube was always used for each washing. The samples containing magnetically immunocaptured cells were observed and counted in a C-Chip disposable counting chamber (Biochrom, Berlin, Germany) using a Nikon Eclipse 80i microscope (Tokyo, Japan). The images were acquired and processed using NIS-Elements AR Analysis 3.2 software (Nikon, Tokyo, Japan). All experiments were performed in triplicate with the MCF7, SKBR3, Raji and/or A549 human cell lines.

### 2.7 EpCAM+ cells immunocapture in Ephesia chip

The PDMS Ephesia chip was prepared according to the procedure thoroughly described by Saias et al. [18]. After the rinsing and equilibrating the inner space with PBS containing 1% (m/v) BSA, the system was ready for the biospecific phase. Thus, the superparamagnetic immunosorbent in equilibrating buffer was flowed into the chip 10 µl/min). When sufficient microspheres were inside the two chambers (capture zones), the magnetic field was switched on (30 mT) and the micropillars ready for EpCAM+ cells capture were formed. The desired number of certain EpCAM+ cells was then flowed into the microfluidic chip. The chamber with anti-EpCAM micropillars was observed using a Nikon TI-E inverted microscope. RAJI cells (EpCAM-) used as a negative control were subsequently flowed into the device to reveal nonspecific adsorption.

## 3 Results and Discussion

### 3.1 Preparation of anti-EpCAM-PEG-PGMA microspheres

*Preparation of hydrazide-PEG-PGMA microspheres*

In our study, the recently developed superparamagnetic poly(glycidyl methacrylate)-based (PGMA) microspheres were used for preparing an anti-EpCAM immunosorbent. Results presented in other works have demonstrated the versatility and easy applicability of PGMA microspheres for use in enzyme immobilization as well as DNA or cells isolation [35, 36, 40, 41]. The polymer microspheres were prepared using a multistep swelling polymerization ending with incorporation of magnetic iron oxide [42]. Such method enables the production of monodisperse highly magnetic particles with stable physical, chemical and biocompatible properties [36]. Based on previous experiments with PGMA microspheres, we had known that their natural chemical composition may lead to nonspecific adsorption onto microfluidic PDMS channels and investigated cells. Therefore, surface coating was seen as a reasonable solution for minimizing this effect.

In our case, we decided to use PEGylation of the microsphere because it provides several important aspects, such as hydrophilicity, biocompatibility, and, moreover, the incorporation of various functional groups such as hydrazide, hydroxyl, amino, sulfhydryl and/or carboxyl onto the microsphere's surface which might be subsequently used to bind the desired ligand. PEG is supplied in various forms: linear or branched, with heterobifunctional (e.g. amino-PEG-carboxyl) or homobifunctional groups (e.g. hydrazide-PEG-hydrazide) [43]. Implementing of hydrazide groups through linear homobifunctional PEG seemed to be advantageous for this purpose, inasmuch as this group allows an oriented type of antibody binding using site-directed covalent coupling of IgG through their carbohydrate moieties after oxidation by sodium periodate [37]. In this way, the antibody can maintain its binding function after grafting [44] and multiple-site attachment or random orientation can be

avoided. Once the PGMA microspheres were coated with hydrazide-PEG-hydrazide, the anti-EpCAM antibody was immobilized (Fig. 1).

*Selection of suitable anti-EpCAM mAb*

mAb clone with optimal affinity to the EpCAM molecule was selected using the dot-ELISA affinity test described by Svobodova et al. [45]. Using this technique, 8 various clones were tested for their affinity to recombinant EpCAM protein and one with high affinity index was selected, HEA-125 (Progen Biotechnik, Germany). Such findings were confirmed using commercial Dynabeads® M-280 Tosylactivated for anti-EpCAM immobilization. Because the covalent binding of IgG molecules are site-directed, e.g. both binding sites of IgG are sterically accessible for the surface molecules of the cell to be captured, it can be assumed that the rate of cell capture efficiency is only a question of mAb capture efficiency.

*Evaluating of the IS quality*

The immunocapture efficiency of such IS was first determined in a batchwise arrangement and then also in an Ephesia chip (Fig.2 A-B). The capture rate in batchwise arrangement was 330 cells, which was highly comparable with the 319 captured cells for Dynal Epithelial Enrich (the microspheres' positive IS control). The IS evaluation in Ephesia chip also showed efficient capture of EpCAM+ cells (MCF7) immediately upon the cells' being put into contact with immunosorbent columns, which means mostly in the first rows of the capture zone (Fig. 2 B). This experiment therefore confirmed the correct selection of mAb clone (HEA-125) with strong affinity for EpCAM.

### 3.2 Evaluation of cells interaction with microspheres

The non-specific interaction of the PGMA microspheres, either PEG-coated (PEG-PGMA) or non-coated (PEG-free-PGMA) with the cells was studied in this experiment. PEG-PGMA microspheres biofunctionalized by nonspecific HuIgG (HuIgG-PEG-PGMA, negative control) and/or anti-EpCAM mAb (anti-EpCAM-PEG-PGMA, positive control) were also included into the study to see the differences between nonspecific and specific interaction with EpCAM+ cells. The tested PGMA microspheres were incubated with the MCF7 cell line in a batchwise arrangement and the amounts of cells captured using various carriers were compared. Fig. 3 A compares the cell capture using various carriers based upon average data from the triplicated experiments. A clear 78% decrease of nonspecific adsorption of PEG-PGMA microspheres vs. PEG-free-PGMA can be seen, and there was only 4% of nonspecific adsorption of cells on HuIgG-PEG-PGMA compared to the specific capture of anti-EpCAM-PEG-PGMA microspheres. In addition, the specific capture rate of anti-EpCAM was comparable (at 329 vs. 331 cells) with that of the positive IS control Dynabeads® Epithelial Enrich.

### 3.3 EpCAM+ cells immunocapture in batchwise arrangement

The anti-EpCAM-PEG-PGMA carrier was also characterized from the immunocapture point of view. The IS was incubated in a batchwise arrangement in triplicate with the cell lines differing in EpCAM density: the MCF7 cell line with high EpCAM expression, SKBR3 cell line with medium expression, A549 with low expression, and Raji without EpCAM expression (negative control). Fig. 3 B shows that both tested immunosorbents, anti-EpCAM-PEG-PGMA and Dynabeads® Epithelial Enrich (the microspheres' positive IS control) had equally high capture rates for all four cell lines (note that the results are averages of triplicate measurements). The SKBR3 and MCF7 cell lines had almost similar results in spite of their

different EpCAM surface density. Probably due to role of steric hindrance the immunosorbent was at his maximum of immunocapture rate in case of MCF7, thus even though the EpCAM density on SKBR3 was significantly lower the capture rate was almost the same. Such results were in accordance to those for the A549 cells, which were captured at a lower rate because of their extremely low EpCAM density. As a negative control, Raji showed that anti-EpCAM-PEG-PGMA IS has a certain rate of nonspecific adsorption (1.9%) compared to Dynabeads® Epithelial Enrich. Considering the fact, however, that the microspheres were developed in a scientific laboratory and not in a specialized company, the microspheres might contain some residues, such as iron oxide on the surface, damaged particles, or other impurities which could cause the nonspecific adsorption of the cells. Micrographs of captured cells surrounded by magnetic anti-EpCAM-PEG-PGMA microspheres are shown in Fig. 3 B.

### 3.4 EpCAM+ cells immunocapture in the Ephesia chip

An Ephesia chip utilizes a microfluidic technology developed for capture of CTCs from the blood of cancer patients. The first concept of the Ephesia chip, where magnetic microspheres were employed in a PDMS channel for B lymphocytes capture, was described by Saliba et al. in 2010 [19]. The self-assembling magnetic bead array took shape in a microfluidic channel on a 0.5 mm (height) × 10 $mm^2$ area and allowed only low throughput of the sample. The design was subsequently improved by Saias et al. [18]. The channel containing a micropost array was replaced by 2 large microfluidic chambers (33 × 3 × 0.5 mm) of diamond-tree shape structure, thereby enabling 100-fold higher uniform flow throughput. Such area of the micropillars ensures contact of the cells with the antibodies immobilized on the microspheres, and thus a high capture rate. In addition, the capture zone for CTCs is thus precisely defined by the array of micropillars. This makes it easy to control where the anti-EpCAM antibodies

are placed and in what density as the microspheres are developed and biofunctionalized outside the chip. Hence, the requirement for stability of the microfluidic device could be significantly reduced while the cost-effectiveness and technological aspects substantially improved in comparison to designs based upon biofunctionalized inner surfaces of the microfluidic device.

The immunocapture efficiency of epithelial cells in the Ephesia chip using the PEGylated anti-EpCAM IS was demonstrated with the MCF7 cell line. First, the IS was made to flow through the chip. When the chamber was filled with microspheres suspension, the magnetic field was turned on (30 mT) and the columns were formed according to the bottom layer array (Fig.4 A-C). The excess IS was removed from the chamber using a PBS with 1% BSA (10 μl/min), and thus only microcolumns resisted (Fig. 4 D). Such capture zone was ready to attach specifically the EpCAM positive cells (MCF7 cell line). A total of 500 MCF7 cells stained by Hoechst were flowed in. When the number of captured cells was determined using a fluorescent microscope, 1 million EpCAM negative cells (Raji cell line) were flowed into the capture zone. The cells were again counted and capture efficiency was estimated. The MCF7 cells were captured mostly in the first rows of the capture zone (Fig. 5). The results showed 65% cell capture efficiency with MCF7 in the Ephesia chip and only 1.2% of nonspecific adsorption.

## 4 Concluding remarks

In summary, a magnetic immunosorbent was developed for EpCAM positive cells capture in an Ephesia microfluidic chip. All required IS characteristics concerning the size of the beads, surface coating, antibody biofunctionalization, capture rate, and other physicochemical properties were fulfilled using PGMA microspheres PEGylated with hydrazide-PEG-

hydrazide, Only the PEG with hydrazide fuctional groups enables to immobilized IgG in proper way to ensure the best Fab binding fragments accessibility and the specific cell capture is dominant. Significantly decreased nonspecific cell adsorption confirms the correctness of our approach. The anti-EpCAM mAb clone HEA-125 was proven suitable for biofunctionalization of the microspheres. The anti-EpCAM-PEG-PGMA carrier preliminary tested in batch was then used successfully in the Ephesia chip.

Perhaps the greatest challenge in developing a device for CTC detection remains in the immense heterogeneity of CTCs. That complicates the isolation and characterization of CTCs [46], as not all CTCs express the EpCAM molecule on their surfaces. Therefore, we might consider in the future turning to alternative surface CTC markers corresponding to the specific type of cancer, such as CD146, which is detected on EpCAM-negative CTCs in the case of breast cancer [47]. The proposed process for immunosorbent preparation is versatile, and the entire microfluidic system is made available for immobilization of mAb with any specificity. Thus, for example, we could come up with a chip having micropillar arrays with different specificities linked in series to captured CTCs of a defined cancer type. Of course, many aspects concerning the characteristics of CTCs will need to be clarified and defined beforehand. We strongly believe that as the progress moves inexorably forward, microfluidics can supply a robust device in the future able to specifically capture, characterize, and even culture CTCs. We also anticipate that a device such as an Ephesia chip may be useful in developing flexible cancer diagnostics and prognostics, and consideration may be given even to devising some hybrid system combining a CTC immunocapture strategy with another based on the physical properties of CTCs.

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Fig.1. Scheme of PEGylation and biofunctionalization of PGMA microspheres with anti-EpCAM. EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EpCAM, epithelial cell adhesion molecule; PGMA, polyglycidyl methacrylate; Sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt.

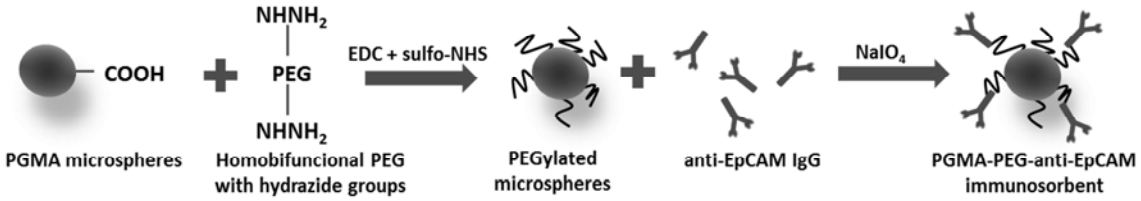

Fig.2 A-B. Micrographs showing immunocapture of MCF7 cell line on magnetic IS (Dynabeads® M-280 Tosylactivated + anti-EpCAM mAb clone HEA-125): A – in batch arrangement (in C-chip) and B – in Ephesia chip. CTCs are captured mostly in first rows (arrows show flow direction of fluid) of the capture zone.

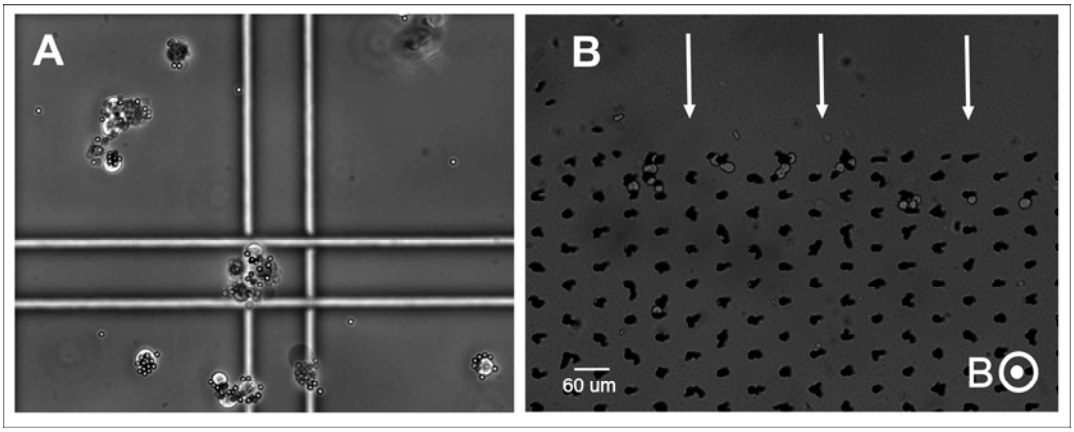

Fig. 3 A-B: (A) Graph shows amounts of immunomagnetically captured EpCAM+ cells (MCF7, average of 5 measurements) using various magnetic carriers: PEG-free-PGMA microspheres (without coating), PEG-PGMA (PEG-coated microspheres), HuIgG-PEG-PGMA, and anti-EpCAM-PEG-PGMA microspheres. (B) Graph shows amounts of immunomagnetically captured cells with various EpCAM density: MCF7 (EpCAM +++), SKBR3 (EpCAM ++), A549 (EpCAM +) and RAJI (EpCAM −) using PGMA-PEG-anti-EpCAM immunosorbent, average of 3 measurements.

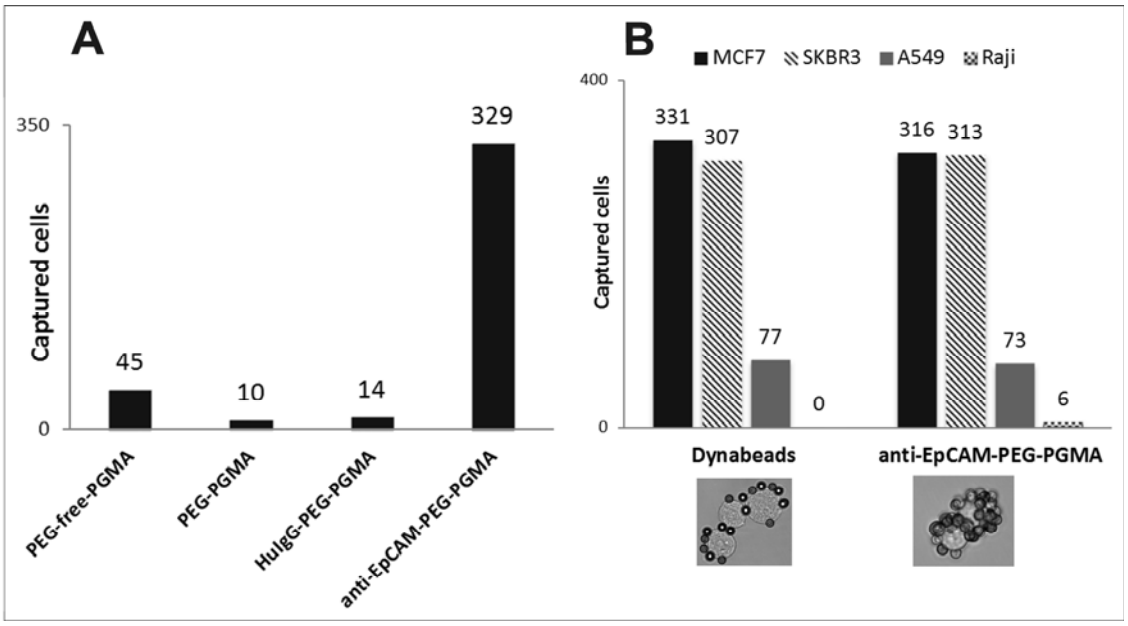

Fig. 4 A-D. Micrographs of the Ephesia chip's inner space – the capture zone: (A) before adding IS, (B) magnetic IS flows inside (10 µl/min), (C) magnetic field is turned on (30 mT), (D) microcolumns are formed and excess of IS is washed away, small figure shows the micocolumns of anti-EpCAM-PEG-PGMA microspheres.

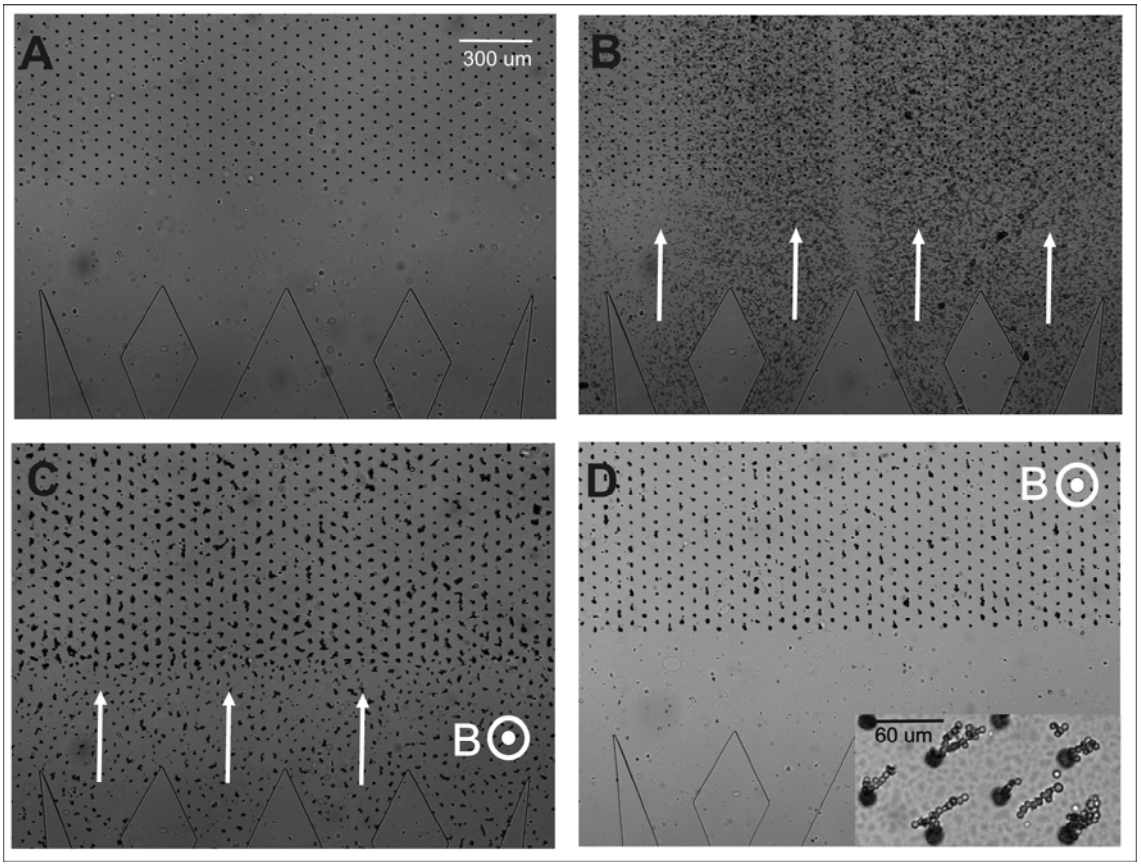

Fig. 5. Micrograph of MCF7 cells captured in Ephesia chip using anti-EpCAM-PEG-PGMA carrier.