Lab-on-chip for the Isolation and Characterization of Circulating Tumor Cells

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ABSTRACT

A consortium of seven European partners has undertaken the challenging task to develop a miniaturized system capable of the isolation and characterization of circulating tumor cells (CTC), which should have its use as a minimally invasive tool for the early, primary diagnosis of cancer, for cancer therapy monitoring and for the detection of recurrent disease. The proposed lab-on-chip will integrate modules for sample incubation, cell isolation - detection and mRNA amplification - detection with state-of-the-art micro-fluidics and micro-engineering. This paper reports on the general concept of the system and elaborates on the development and first test results of the different modules and their integration.

Keywords: lab-on-chip, circulating tumor cells, immunomagnetic separation, multiplexed amplification, biosensor arrays

1 INTRODUCTION

Circulating tumor cells (CTC) are tumor cells that invade the bloodstream and cause distant metastases. Recently, the levels of CTC detected in metastatic breast cancer patients before and after therapy were shown to be significant predictors of progression free and overall survival [1]. The very low levels of CTC in PB, *i.e.* down to less than 1 cell per mL, make their detection very challenging. The standard method for the detection of isolated tumor cells (ITC) has long been Ficoll-Hypaque density centrifugation followed by direct immunocytochemical (ICC) methods [2]. To be able to analyze higher ITC numbers, numerous cell enrichment methods have been developed based on either immunomagnetic separation (IMS), *e.g.* Dynabeads (Invitrogen), or flow cytometry [3]. Combining IMS and ICC, the CellSearch System (Veridex) is designed for circulating tumor cell (CTC) detection in peripheral blood (PB) [1]. More specifically, this semi-automated system first performs positive IMS using anti-EpCAM coated ferrofluids. The enriched cells are then permeabilized, fixed, immunostained and identified as tumor cells by their positive cytokeratin and nuclear staining and negativity for CD45. The drawback of this system is that it still needs visual confirmation. A promising alternative is the combination of positive IMS with quantitative RT-PCR [4]. In addition, positive IMS using multiple traction antibodies and quantitative RT-PCR using multiple markers would help to overcome the background signals from normal PB [5].

To facilitate the detection of ITC in different sample matrices and allow their further clinical evaluation in an automated fashion, systems that allow the sequential ITC enrichment, quantification and thorough characterisation of detected cells would be of great value. Further addressing the future health care requirement aiming at individualized diagnostics-based therapy, lab-on-chip systems for the detection of CTC from PB of breast cancer patients could be minimally invasive tools to be used at the point-of-care or point-of-clinic.

2 GENERAL CONCEPT

The proposed lab-on-chip will integrate the different analytical steps of the isolation and characterization of CTC in PB, focusing on the use of positive IMS with quantitative RT-PCR using multiple markers (Figure 1):

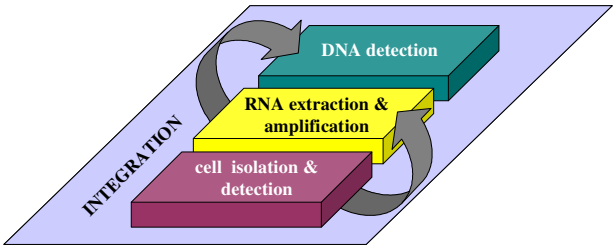

Figure 1: Schematic presentation of the different operations to be performed by the lab-on-chip for the isolation and characterization of rare cells.

Four microsystem modules are being developed for:
1) sample incubation and immunomagnetic isolation;
2) cell isolation and cell counting;
3) cell lysis, RT of mRNA and amplification;
4) detection of amplified DNA.
These different microsystem modules will then be integrated and supplemented with state-of-the-art microfluidics and micro-engineering to come to a stand-alone device.

In parallel, antibodies are being developed for a selection of CTC surface markers to allow for improved IMS; and, a panel of mRNA markers has been identified to allow for a thorough characterization of the isolated CTC.

3 DIFFERENT MODULES

3.1 Sample incubation

The first module of the lab-on-chip system is the sample incubation module. It is designed to be able to hold a high sample volume (over 15 mL) and will allow insertion of PB (holding CTC), addition of antibody-coated beads, mixing of the sample, attraction of the beads and bead-cell complexes, removal of the PB and addition of washing buffer. After IMS and washing, the beads and bead-cell complexes are collected on a magnet and moved to the second module, with a volume of about 100 µL.

3.2 Cell isolation and counting

The device for the isolation and counting of CTC, following IMS, comprises four current-carrying conductors composed of repeating ring structures along the separation direction [6]. By sending alternating and periodic current to the four conductors, a traveling magnetic field gradient is produced allowing the transport of both the bead-cell complexes and excessive beads. Due to their different magnetophoretic mobility, the bead-cell complexes will have a different velocity and can be separated from free beads. Combining such an isolation device with an H-shaped fluidic channel allows to specifically force the beads-cell complexes into the detection channel, where they are counted by flowing them over a spin-valve sensor (Figure 2).

The separation ability of these devices has been proven with a mixture of two different types of magnetic beads [6] and with a mixture of Molt-4 cells and anti-CD45 coated beads.

3.3 Cell lysis and mRNA amplification

Following isolation and detection, CTC have to be lysed and their mRNA has to be extracted and amplified before proceeding to detection. Because of its unique multiplexing capabilities, Multiplexed Ligation-dependent Probe Amplification (MLPA) has been chosen as the basis for this module [7]. Assays are designed for the multiplexed amplification of up to 50 specific cancer markers expressed in breast carcinoma from single or a few CTC isolated from PB. The assays consist of three major steps;
1) RT followed by multiplex cDNA pre-amplification;
2) simultaneous hybridization of the all MLPA probes;
3) ligation and amplification of hybridized MLPA probes.
The use of three oligonucleotides in the hybridization step and of only one universal primer pair in the MLPA amplification step allows the detection of changes in relative expression (Figure 3). Asymmetric PCR will be applied to obtain ssPCR product.

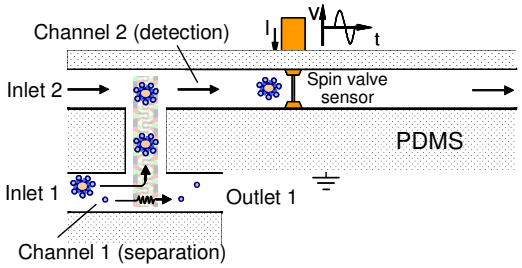

Figure 2: Cell isolation device bridged between two channels. Cell-bead complexes are moved towards a detection channel, while free beads are flown to the outlet.

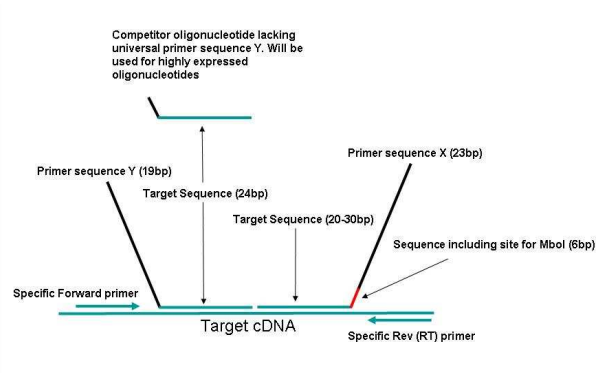

Figure 3: MLPA probe design to allow the detection of relative mRNA expression.

The microsystem for cell lysis and mRNA amplification is being developed for the assay outlined above, based on an intelligent design of heating elements, active cooling principles and fluidic channels and their actuation.

3.4 Detection of amplified cDNA

In the detection module, the amplified cDNA will be detected using an array of electrochemical sensors. Detection strategies are being assessed in which the ssPCR product is sandwiched between a probe immobilized on the electrode surface and a reporter probe bearing an enzyme

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label. In addition, liposomes encapsulating hundreds to thousands of enzymes are being investigated as a means of signal amplification and improved sensitivity. This labelled sandwich assay detection strategy is being compared to label-free impedimetric detection and to approaches using molecular beacons. Currently, mainly the electrode surface chemistry, the sandwich assays, the liposome synthesis and detection signature of the electrochemical labels are being characterized and optimized.

For the multiplexed detection of the MLPA end-product, arrays with 64 individual electrodes have been designed and are being fabricated. Different fluidic designs and fluid actuation mechanisms are being tested. Such a biosensor array would allow the simultaneous detection of 25 target markers, including duplicates and controls.

4 INTEGRATION

Bringing together the individual modules the system set-up will be built up as shown in Figure 4. Three syringes on top of the system are used for sample delivery, as reservoir for washing buffer and as waste reservoir for sample and washing buffer.

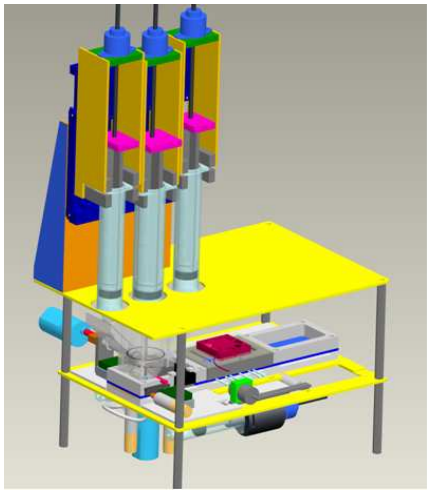

Figure 4: Cartoon of the integrated lab-on-chip device and system set-up (©IMM, 2006).

5 CELL SURFACE AND RNA MARKERS

To allow for improved IMS, antibodies are being developed for a selection of CTC surface markers and their performance will be compared to the commonly used antibodies for EpCAM, MUC-1, Her-2 and CEA.

A panel of about 20 mRNA markers has been identified to allow for a thorough characterization of the isolated CTC. The markers have been selected such that the panel allows to identify the epithelial origin, to differentiate benign and malignant cells, to describe the tissue origin and/or histological type, and to aid with therapy selection.

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