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Is a real-time quantifiable liquid biopsy achievable using a microfluidic lab-on-chip ?

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Abstract

An increasingly relevant functional measurement is a liquid biopsy to assist in the diagnosis of cancers. The existing approach for liquid biopsy is to utilize microfluidic chips for the isolation of circulating tumor cells (CTCs) or exosomes or extracellular vesicles (EV) from patient samples, and then for the analysis of the cargo contained inside the CTCs, exosomes or EVs. However, such an analysis does not provide a real-time liquid biopsy, since there is a long delay between the time of sample collection and the results from the analysis. Microfluidic chip-formats also provide the capability to mimic tissue functions from the analysis of small numbers of cells cultured in the chip. Analysis of the secreted molecules from such cells could provide a measurement of the secretome, which could be analogous to a liquid biopsy. A 3D structural organization of cells in microfluidic chips is usually in the form of organoids or spheroids. The analysis of organoids or spheroids is well-adapted for immunohistochemistry or ELISA-type identification of surface markers, but not for real-time analysis of secreted molecules since the fluid and molecules in the interior volume of the organoid or spheroid is not accessible in real-time. We have recently proposed an alternative novel design for a microfluidic chip format comprising 3D micro-niches that provide a real-time analysis of secretions produced directly from small numbers of cells. The microfluidic chip with 3D micro-niches then analyses the secretions from these monolayers in real-time ("secretome"). The microfluidic chip includes electronic biosensors that provide real-time measurement of secreted molecules. This short review concludes with a proposition for the means to utilize this novel microfluidic chip to function as a real-time and quantifiable diagnostic screening device to differentiate cancerous cells from healthy cells.

Keywords: 3D microenvironment, cell monolayer, microfluidic, lab-on-chip, secretome, real-time measurement

Introduction

Monolayer (in 2D) cultures have been very useful models for gene discovery and early work on viral transformation, but such 2D systems do not provide a cell-model that replicates the architecture of exocrine gland tissues [1]. Cell models that replicate exocrine gland architecture are important tools in the search for drugs to combat disease and cancer. For example, cancer of breast tissue is a significant cancer amongst women. Vidi et al 2013 describe methods for the growth of breast epithelial cells into physiologically relevant 3D cell culture, which demonstrate the important roles that cell polarity, cell shape and the development of proper basement membranes play in the physiology of exocrine tissues in the breast. Such physiologically relevant 3D cell culture models of breast tissue are important tools in the search for therapeutic agents to fight disease and cancer of the breast tissue [1]. For men's health, the exocrine prostate gland tissue is a significant cause for cancer and other disease. Prostate epithelial cells grown in micro-spheroid cultures in a Microwell-mesh 3D culture system responded appropriately to drugs used to treat prostate cancer [2]. The use of organoids, as 3D culture models would provide even closer tissue-like conditions for studying drug reaction [3]. Those types of 3D cell-culture systems provide information of cell responses that are measurable by imaging or spectroscopic techniques. The limitation of those 3D epithelial models for

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acinar structures is that it is not possible to access the luminal secretions to measure physiological responses in real-time and to identify new cancer biomarkers.

Liquid Biopsy

A relevant aspect for the analysis of luminal secretions is the concept of liquid biopsy. To date, tissue biopsies are core components for cancer patient management to diagnose, assess disease stage, and prescribe appropriate therapeutic regimens. However, tissue biopsies are not only invasive and risky but they fail to provide a temporal insight into the evolution of tumors, and do not fully reflect intra-tumor heterogeneity. Metastatic process occur when cancer cells detach from the primary tumor and invade the blood circulation. Then, these circulating tumor cells (CTCs) can extravasate and colonize distant sites, leading to secondary tumor(s). CTCs are released into the bloodstream from primary and metastatic tumors, and provide a promising clinical potential for information relevant to cancer diagnosis and prognosis. The isolation of CTCs directly from a blood test is referred to as "liquid biopsy" [4]. The state-of-the-art in liquid biopsy is to collect either circulating tumor cells (CTCs), exosomes, and then harvest the liquid inside the CTCs or exosomes for further biochemical analysis. Biochemical analysis of whole-fluid samples is also utilized for liquid biopsy (e.g. whole-blood, complete urine, complete saliva). Urine is easily collected non-invasively without the need for a health-care practitioner. However, the composition of urine is very variable, making biochemical analysis of whole-urine very difficult to interpret due to the heterogeneous composition of urine and the lack of precise and standardized protocols for collection and analysis.

The technical challenges for the isolation of CTCs are the following [4]:

(i) a morphological similarity with some WBCs, reducing the effectiveness of size-based sorting;

(ii) a phenotypic heterogeneity, which makes the use of biomarkers more complex and limits the effectiveness of biomarker-based separation;

(iii) the epithelial-to-mesenchymal transition (EMT) that CTCs can undergo, which results in a decreased expression of epithelial markers and the appearance of a mesenchymal phenotype, which is associated with an increase in the capacity of invasiveness, immune escape, and metastasis;

(iv) difficulty in preserving the integrity of CTCs isolated for downstream characterizations.

To date, the Cellsearch™ (Veridex, Raritan, NJ, USA) system is the only device approved by the US Food and Drug Administration (FDA) for CTC detection and enumeration for clinical use. Since its introduction in 2004, the CellSearch™ system has been used as a diagnostic and prognostic tool in patients with metastatic breast, colorectal, and prostate cancer. Although this system is considered a "gold standard" for CTC detection, it has several drawbacks. The detection sensitivity of this approach highly depends on epithelial markers, which results in a low recovery for cells that underwent EMT. Cellsearch™ has a CTC recovery rate of only 2% for mesenchymal breast cancer cell lines. In addition, the system enriches CTCs with a high background of contaminating WBCs, leading to low purity (0.01–0.1%) and hence limiting further analyses [4].

Microfluidic Platforms for Liquid Biopsy

Microfluidic devices possess unique advantages over conventional appr oaches, among which one can cite the following:

(i) their micrometric dimensions and laminar flow nature, enabling precise object manipulation and single-cell study;

(ii) the handling of small volumes, which facilitates the analysis of rare or expensive samples and speeds up the processes, leading to cost-effective devices;

(iii) the integration of various functions (mixing, focusing, sorting, trapping, detection, etc.) into a single device, leading to compact and portable systems, and therefore opening the way for the implementation of point-of-care devices.

Nonetheless, the state-of-the-art techniques require a complex sample collection and purification step, followed by a complex biochemical analytical step. The complex analytical step is required to differentiate the types of molecules that are secreted from the tissues of the body. More recent technical approaches have utilized microfluidic lab-on-chip devices are used to achieve those steps. Microfluidic devices that have been commercialised for CTC isolation utilise technologies that :

(i) rely on the difference in CTC size and deformability compared with blood cells ;

(ii) rely on the difference in other physical properties, such as density and electrical charges ;

(iii) rely on the biological properties of CTCs and exploit their specific surface marker expression.

Commercialised microfluidic devices that rely on physical properties include Parsortix® (ANGLE plc, Surrey, UK), ClearCell® FX1 (Biolidics Limited, Mapex, Singapore), VTX-1 (Vortex Biosciences, Menlo Park, CA, USA), and ApostreamTM. Commercialised microfluidic devices that rely on biological properties of CTCs include HBCTC-Chip, NanoVelcroChip, MagRC, μ-Mix-MACS Chip, and LPCTC-iChip.

Despite the advances in different enrichment protocols that use microfluidic devices to isolate CTCs from the blood, the analysis of CTCs is still a technical challenge. The analysis is usually a two-step procedure, consisting of enrichment and detection, both being critical for the specificity and sensitivity of the overall approach. Regardless of whether CTCs are detected and further characterized by their intracellular proteins, gene expression pattern or genetic make-up, the background of benign cells may interfere with the analysis and thus needs to be substantially reduced. Such benign cells that are particularly relevant are the residual white blood cells (WBC), because a number of genes may also be expressed in leukocytes and hence will interfere with the analysis of the CTCs, particularly at the transcriptional level [5].

Microfluidic Devices that Incorporate Biosensors for Analysis of Secretions from Single Cells

The secreted proteins, peptides, metabolites, and microvesicles from cells can participate in cell signaling and cell-to-cell communication, mediating a broad range of responses on target cells, such as migration, proliferation, and differentiation. The ability to detect secreted products can have potential clinical applications by employing them as molecular biomarkers for disease diagnosis, prognosis, and therapy evaluation. Cedillo-Alcantar et al (2023) report a microfluidic device that pairs single cells and antibody-functionalized microbeads in hydrodynamic traps to quantitate cytokine secretion. The device contains 1008 microchambers, each with a volume of ~500 pL, divided into six different sections individually addressed to deliver an equal number of chemical stimuli. Integrating microvalves allowed us to isolate cell/bead pairs, preventing cross-contamination with factors secreted by adjacent cells The Cedillo-Alcantar et al (2023) device utilises fluorescence sandwich immunoassay as the biosensor on the microbeads. The fluorescence output signals required imaging using a specialised microscope, followed by image analysis that was not fully automated [6]. Although the Cedillo-Alcantar et al (2023) device utilised a biological cell in a microfluidic system to provide a signal, the method of analysing the signal was not adapted to real-time biosensing of the response from the cell. Another aspect of the state-of-the-art is to utilize organoids or spheroids prepared from target cells that ware then maintained inside microfluidic systems.

Despite the benefits of real-time biosensing, the most widespread analytical techniques employed in microfluidic systems have been mostly inherited from molecular biology and histological analysis which rely mostly on post facto endpoint assays. These techniques include daily observation under an inverted microscope, which is subjective and qualitative; labour intensive and large volume off-chip assays (e.g., ELISA, HPLC, or mass spectrometry); and endpoint assays that terminate the culture like cellular staining with fluorescent or colorimetric labels, tissue fixation and sectioning for histological studies or cell lysate offline analysis [7]. Real-time biosensing using electrochemical biosensors has been reported for measurements of oxygen, lactate, and glucose [8]. However, the major difficulty remains for organoids in microfluidic systems that the secretions into luminal structures from the interior of the organoids is not possible with existing

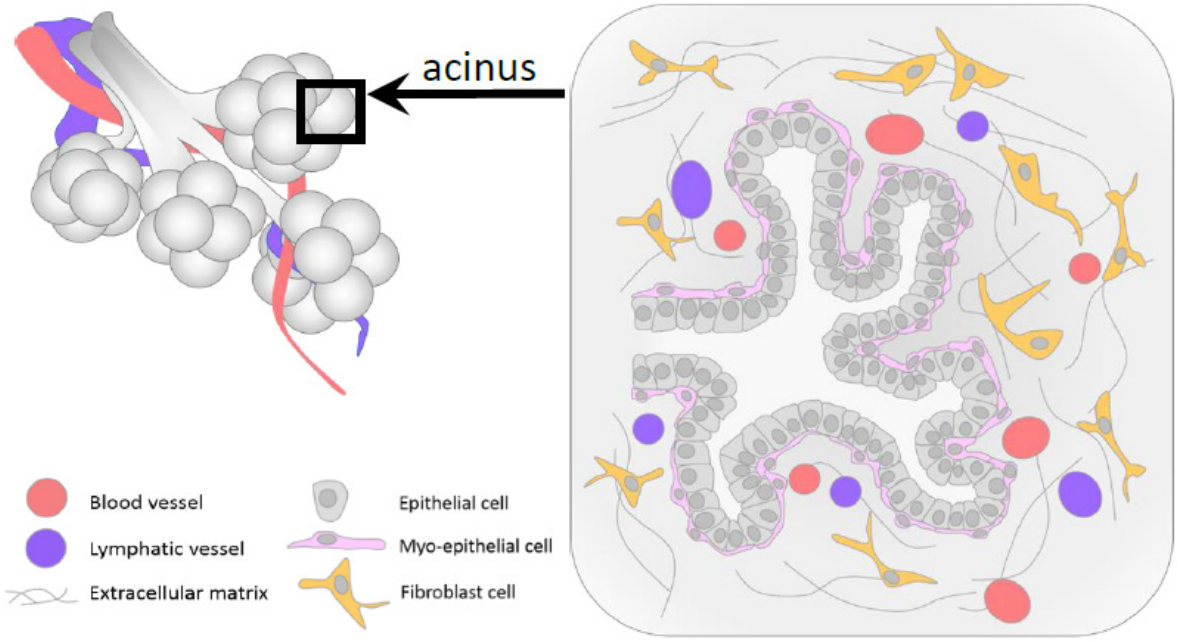


Figure 1. Scheme of glandular tissue that forms common structural and functional acini and duct units. Enlarged scheme of a lumen-based glandular tissue, surrounded by extracellular cues. Reproduced with permission from [1].

state-of-the-art organoid microfluidic systems.

Our concept for 3D micro-niches inspired by acini from exocrine glands

The need for 3D differentiation-relevant model systems is particularly well exemplified for generating functional glandular tissue units and investigating carcinoma, which is a major form of cancer that originates from the malignant transformation of epithelial cells. Exocrine glands form elaborate architectures consisting of ducts and acini lined with epithelial cells (Figure 1).

The 3D micro-niches in our microfluidic chip (LoC) were designed to recapitulate the structure of an acinus connected to a collecting duct [10,11]. The form of the 3D micro-niches are inspired from acini and the need to have a collecting channel for the secretion from the 3D micro-niches that is connected to the perforation in the LoC below the 3D micro-niche (Figure 2).

Our LoC design provides the advantage of a microstructured 3D microenvironment, which is different to existing compartmentalized platforms that are composed of two microfluidics channels separated by a horizontal thin porous membrane, with cells being cultured on one side or both sides of this membrane. Our approach has recently been highlighted [12]. In existing chip-formats, such a porous membrane is made from an elastomeric materia that can be stretched in a cyclic way to model surface strains that cells would be exposed to in vivo, for example, in the lung–alveola barrier [13] or the

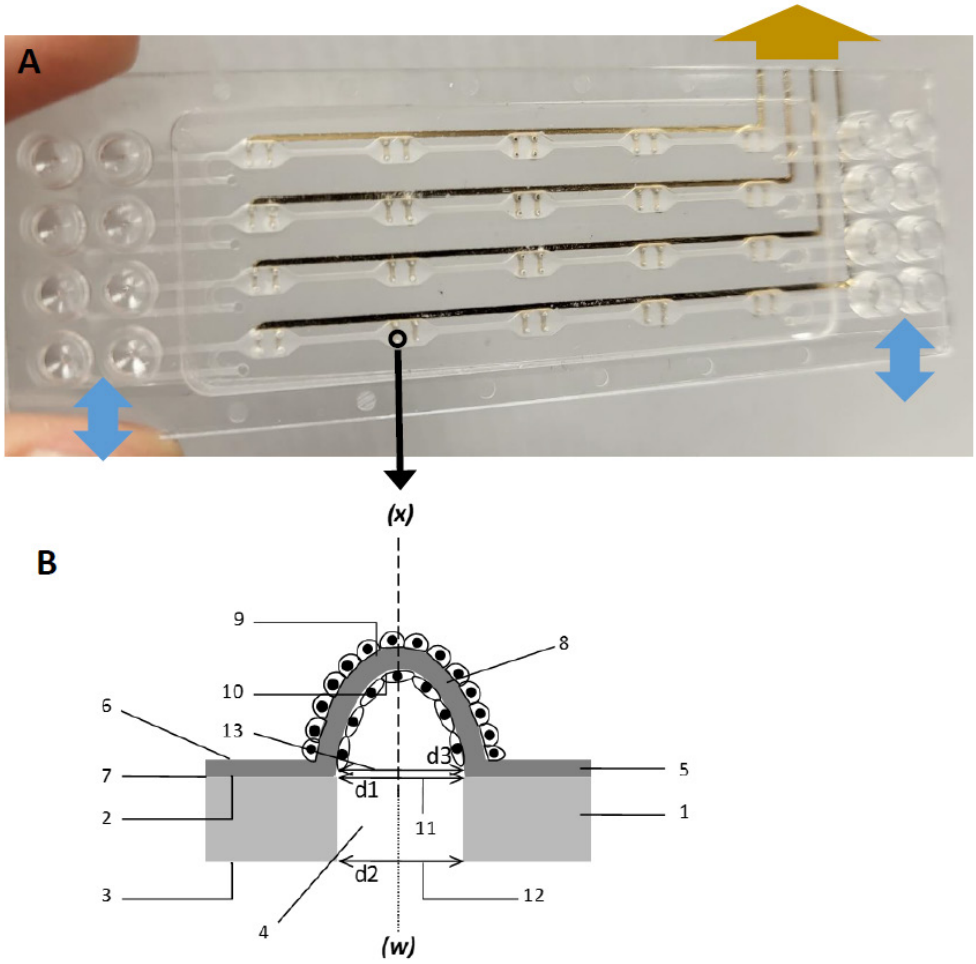


Figure 2. (A) Lab-on-chip format (LoC) of the 3D micro-niches connected with microfluidics and including gold tracks for electronic biosensors. This LoC is constructed from polycarbonate. The gold arrowhead indicates the output for electronic biosensors. The blue arrowheads indicate the input/output connectors for fluids, cells and microcapsules. The black circle indicates the position of one of the 3D micro-niches. **(B)** Schematic cross-sectional view of a 3D micro-niche, which comprises a 3D protuberance (8) formed in a nanostructured permeable membrane (5) to cover a perforation in the support (4, 11). The nanostructured permeable membrane is tightly connected to the material of the LoC (2). The inner side of the LoC material is the wall of the lower microfluidic channel (3). The perforation in the supporting LoC material (1) can be of a cylindrical shape (d1 = d2), but it is preferred to be in a conical shape (d1 < d2). The inner face of the protuberance (2) is covered by an assembly of a first cell type at the stage of the confluence (10), and the outer face of the protuberance (6) can be covered by an assembly of a second cell type at the stage of the confluence (9). Reproduced with permission from [13,14].

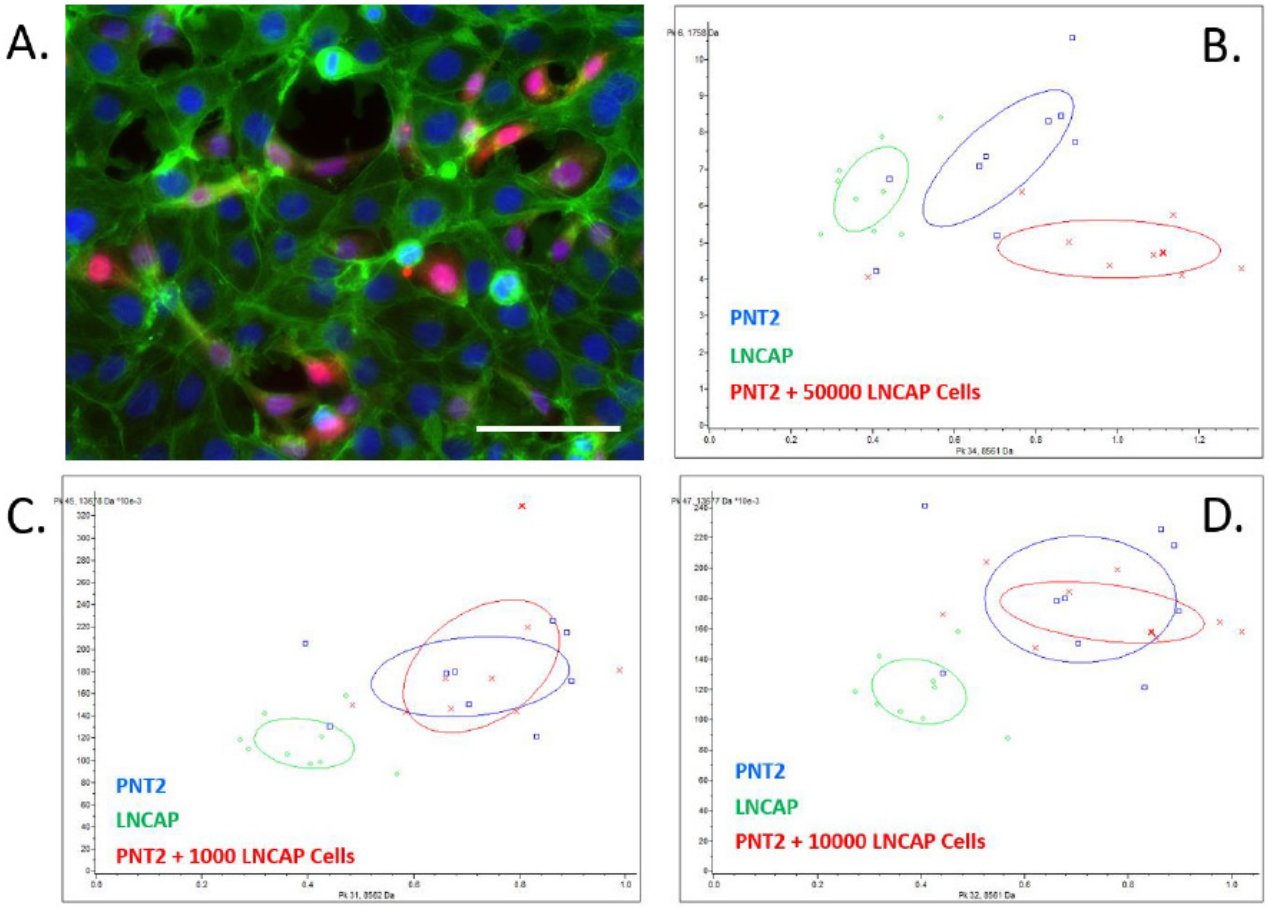


Figure 3. PNT2 cells co-cultured with LNCaP cells. **A.** Mixed population of non-cancerous (PNT2) and cancerous (LNCaP) prostatic cells. Confocal image of LNCaP labelled with CellTracker™_Green (here in red) on a PNT2 cells layer (here in green) cultured for 72h. Blue: Hoechst; Green Phalloidin; Red: LNCaP labelled with CellTracker™_Green. False colors are used for better visibility. Bar : 100 µm. **B,C,D.** 2D peak distribution of MALDI profiling analysis of PNT2 cells co-cultured with LNCaP cells. The cell types are colour-coded and the number of LNCAP cells seeded in the co-culture are indicated. proportions of the Reproduced with permission from [11].

intestine barrier [14]. Cardiac 3D constructs (heart-on-chip) embedded in a gel and set in between two compartments have been also developed [15]. Liver-on-chip models show great promise for investigating drug bioactivation, drug clearance, susceptibility to drug-induced liver injury, nanoparticle or chemical toxicity, and production of reactive metabolites that can interact with other organs [16-18]. However, it has still not been possible to integrate a functional biliary outflow tract into these devices [17]. Other challenges must also be overcome for these platforms such as identifying alternative materials to polydimethylsiloxane, or integrating in-line sensors to monitor the cell microenvironment or analyze cell secretions, feedback controls, and real-time imaging [17], while preserving a good balance between complexity and practicality [19]. Although there have been outstanding recent successes, to date, 3D epithelial models for acinar structures lack physiologically relevant lumen structures, which hampers the access to secretions and hence the identification of new cancer biomarkers.

Sensitivity of our LoC concept for infiltrating cells demonstrated in 2D culture

The 3D micro-niche microfluidic LoC provides a system to measure the secretome in real-time directly from cells in the micro-niches. This secretome provides a readout that allows this LoC to be used as an in vitro diagnostic device. An example is to detect the presence of infiltrating cancer cells into a healthy monolayer, as we have described for detection of cancers of the urogenital tract [10,11]. To assess the ability to differentiate a healthy PNT2 culture from a PNT2 and LNCaP co-culture, different numbers of LNCaP cells (1,000, 10,000 and 50,000) have been seeded in a PNT2 culture and MALDI profiling analysis has been performed on the supernatant. We first observed that spiked cancerous cells were properly

incorporated in the non-cancerous cell monolayer (Fig. 3.A). Tumour cells settled down, proliferated, and gradually populated the device, to possibly replace healthy basal cells and eventually affect the overall secretory profile of the tissue. Fig. 3.B-D illustrates the 2D peak distribution of MALDI profiling of supernatants from PNT2, LNCaP and PNT2 loaded with 1,000, 10,000 or 50,000 LNCaP cells. While LNCaP culture is clearly separated, the differentiation of PNT2 and PNT2 co-cultured with LNCaP cells is less obvious. However, there is a tendency to separate the PNT2 co-culture condition when the amount of LNCaP is increased to 50,000 cells (Fig. 3.B). This is a ratio of ca. 3 % of infiltrating cancer cells (LNCaP) to cause a measurable changed in the type of secretions from the co-culture.

The number of healthy cells in the confluent monolayer attached to the permeable membrane of the 3D micro-niche is much fewer than for these 2D culture plates. The 3D micro-niche contains around 150 to 250 healthy cells in the attached monolayer. Taking the ratio of around ca. 3 % as the threshold to observe a change in the secreted molecules from the co-culture, then each 3D micro-niche provides the sensitivity to detect 5 to 8 infiltrating cancer cells based on the influence of these infiltrating cells to alter the secretions from the co-culture in the 3D micro-niche.

Proposition for our novel microfluidic chip to differentiate cancerous cells from healthy cells

Our 3D micro-niche LoC concept provides a system that is capable to function as a real-time and quantifiable diagnostic screening device to differentiate cancerous cells from healthy cells. The clear difference from the state-of-the-art is that the approach we have developed using the 3D micro-niches does not require a complex sample collection and purification step in order to perform an analysis of secretions from cells in real-time. Moreover, the 3D micro-niche requires only a few hundred cells to form a monolayer. The 3D micro-niches also have advantages over organoid-based microfluidic systems, by (i) providing the means to collect and sample the secreted molecules from the monolayer of cells, (ii) providing the means to maintain the cells alive for ongoing real-time collection and analysis of the secreted molecules, and (iii) providing the means to utilize several different types of biosensing elements for different type of real-time analytical measurements of the secreted molecules. For the proposed diagnostic LoC device, the intact living test-cells are positioned in the 3D micro-niches using a microfluidic channel, and our 3D micro-niches provide an effective means to obtain a liquid biopsy. Moreover, we are able to position the cells specifically in the 3D micro-niches without the need for an external 4D bio-printing system.

In conclusion, our proposed system would function as a quantifiable screening device by providing a measurement of the secretome "fingerprint" from the monolayer of cells in the 3D micro-niches, which mimic the natural environment of cells in acini. The LoC would be first primed with a monolayer of cultured epithelial of endothelial cells to provide a baseline of healthy cells. Test cells obtained from a patient sample would be introduced using the microfluidics to integrate into the monolayer of control-cells, which is a process that will take the time for the test-cells to first adhere and then integrate into the layer of control-cells. During these processes there is a continuous readout of the secretome from the micro-niches. There will be no change in the secretome if the patient test-cells were healthy. If the test-cells from the patient were cancerous, then there will be a quantifiable change in the secretome induced by the integration of the patient test-cells. Such a change in the secretome will provide a quantifiable measurement for the clinician on which to base further more detailed analysis of the the source and treatment of the cancer detected in the patient cells [10,11].

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