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ABSTRACT

This review covers several aspects of microfluidic devices used for culturing and monitoring of both adherent and non-adherent cells, including a multitude of applications. A comparison of available platforms with high throughput analysis, automation capability, interface to sensors and integration, is reported. Aspects, such as operational versatility of the devices, are scrutinized in terms of their analytical efficacy. It is found that due to multi-functionality capability of modern microfluidics, there is big amount of experimental data obtainable from a single device, allowing complex experimental control and efficient data correlation, particularly important when biomedical studies are considered. Hence several examples on cell culture and monitoring are given in this review, including details on design of microfluidic devices with their distinctive technological peculiarities.

1. Introduction

Over many years, cell cultures have been carried out in flasks, petri dishes or microtiter plates, bioreactors, with solutions that preserve the biosamples alive for the necessary time, typically in the range of days [1–4]. However, cell-environment interactions, which determine cell functionalities and phenotype in vivo and in vitro affecting their responses to stimuli, are not easily replicated or controlled in these traditional formats. In conventional processes, cells are cultured: – often in static condition, where the dynamic physiological conditions of the cells cannot be reproduced; − in conditions where, usually, there is no dynamic monitoring of the physiological parameters; − in presence of unwanted gradients of temperature and CO_2 concentrations. In addition, these protocols require complex manual work causing cellular and metabolic stress and usually imply high costs. In microfluidic devices, the aim is to create more in vivo like systems, to avoid influences on cells functionalities as they are in nature. This can be done by controlling the microenvironment (e.g., cell matrix, flow rate, chemical gradients, pH, temperature, …) and in microfluidic devices is easier than in traditional instruments. Microfluidic technologies for cell-based assays have the potential to increase the biological relevance of cell models while maintaining or increasing the throughput of current methods.

With the advent of microfluidics, cell culturing gained strong innovation in manipulation and preservation of biosamples (Fig. 1). In particular, we refer to cell culture, cell separation and cell assays, where precise control over small volumes of fluids, faster analysis, high throughput, flexible automation and integration capabilities, are necessary [5–8]. Microfluidics provides new devices suitable to investigate complex constructs with accurate and controllable biochemical/biophysical environments by high resolution spectroscopies and real-time imaging. Various types of cells, like adherent and non-adherent, have been cultured in microfluidic platforms [9–11] with the strict requirement of stable cellular microenvironments. Several novel discoveries on cell structures, characteristics and behaviors, have been shown in the literature with the support of microfluidic platforms [12–14].

Unlike conventional cell culture methods, microfluidic-based cell cultures deliver several attractive features with continuous nutrient supply, waste removal, flexibility of schedules, liquid handling systems and high automation capability. The less consumption of fluids, low volumes and therefore the reduced time and cost of analysis make these systems particularly interesting for cell-based assays [15–17]. Hence, microfluidic systems are progressively being used as versatile tools and viable alternatives to traditional approaches. These studies have produced a crucial impact on understanding the basics of those cellular activities which play a key role in determining physio-pathological cell states (e.g. disease characteristics and responses to stimuli). [18–20]. The main goals of cell culture platforms are to mimic closely the in vivo cellular microenvironments and to maintain simplicity for reproducible

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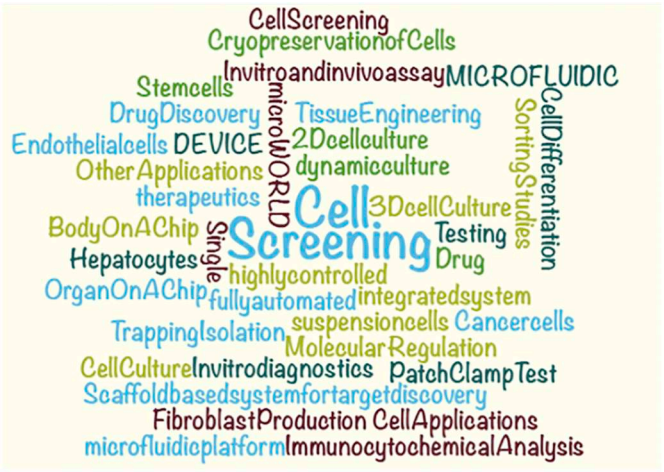

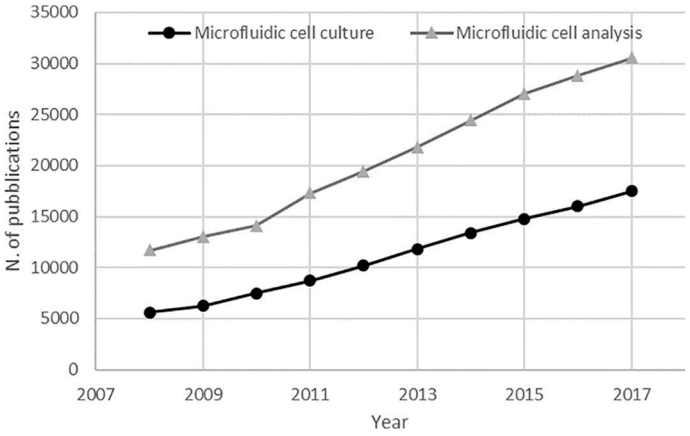

Fig. 1. left: A word cloud listing several applications of microfluidic platforms; right: number of publications in the last 10 years regarding microfluidic cell culture and analysis (source: google scholar).

results [21]. In addition, several parameters need to be monitored on the samples (like pH, oxygen, CO_2, temperature, osmolality and pressure, shear stress) for further downstream investigation. Since the advent of microscale total analysis system (μTAS) in 1990 to the recent integrated microchips, it is evident that the effort to develop futuristic and innovative microfluidic platforms is still evolving, in order to match high-sensitive and high throughput requirements. Moreover, cells of general interest were studied by mimicking both dynamic and static environmental conditions, and further combined with 3D cell culture platforms that represent a breakthrough for in vitro cell growth procedures [22].

Microfluidic platforms applied to tumor cells [23–25], stem cells culture [26–32], and other types of cells [33–36] are designed for several specific applications, such as: single-cell studies [37–39], cell trapping [40,41], filtration [42,43], cell rolling and investigations [44–46], detection of biomarkers [47–49], drug screening and discovery [50–52], organs-on-chip [53–62], body-on-chip [63–65], tissue engineering [66–68], cryopreservation of cells both adherent and suspended cells [69,70].

There are different models available for cell culture today, namely: 2D or 3D static cell cultures, and 2D or 3D perfusion microfluidic cell cultures. Each model shows specific characteristics as shown in Fig. 2. When passing from 2D to 3D cultures, there are many changes in cellular growth behaviors, from cellular shape and architecture to cell-cell adhesions organization, mainly due to the different cellular microenvironments.

Clearly, in vitro 3D cultures better mimic the in vivo conditions of cells inside organs and tissues. Compared to 3D static cultures, microfluidic aided 3D cultures can better face some relevant topics, such as multi-cellular complex environments, suitable cellular vasculatures, and a time-continuous medium exchange instead of a time-discrete exchange. All these issues make 3D cultures as close as possible to in vivo organs. Recently many efforts in microfluidic 3D cell cultures have been driven towards vasculature models, brain and liver models, or to achieve improvements in cancer models (above all lung and breast cancers) [71]. As a few examples, liver-on-chip models have been developed by combination of microscale hydrogels embedding hepatocytes and fibroblasts with a microfluidic chip for cell culture [72], a microfluidic 2-channels architecture separated by porous membranes has been successfully employed for a kidney-on-chip model culturing kidney tubular epithelial cells [73], changes in membrane protein expression of ovarian cancer cells have been observed in 3D cultures along with an increased chemo resistance with respect to the 2D counterpart cells [74], microfluidic platform allowing 3D networks of active neurons along with glial cells have been used for high throughput parallel evaluation of drug effects monitoring the neuronal activity by calcium imaging [75].

A review on the available microfluidic devices with variations in the design, used sensing elements and automation level, is presented here. Versatility in the available microfluidic systems is compared with the degree of automation and multiple analysis capability of the devices. A versatile easy-to-use system is mostly awaited from the microfluidic research community for smooth and straightforward handling by non-technical end-users.

2. Adherent and non-adherent cells

The cellular microenvironment can be broadly classified into three categories holding the key for self-renewal and differentiation of cells: biochemical, physical and physicochemical (Fig. 3) [21]. Cells are widely categorized based on the size, characteristics, occurrence, components, activities, and so on. Cells can be grouped also based on their characteristic to anchor onto the substrate, adherent and non-adherent cells. Those cells that require a substrate to attach and proliferate are adherent cells (anchorage-dependent) and those cells that can grow in suspension are non-adherent cells.

The design of an in vitro cell model system has to focus on cell interactions with their cultivation substrate and its influence and control over the cultured cells. Cells proliferating in a stationary environment (steady state) maintain a time-constant size distribution, and this has a great influence on growth rate of both adherent cells and non-adherent cells [76]. The characteristics of the substrate determine the quality of cellular attachment on the substrate itself. The dependence on the substrate has to be taken into account in the design and fabrication of a multiple variation microfluidic platform when considering either adherent or non-adherent cell growth. A key factor in the proliferation and uniform growth of adherent cells is the hydrophobicity of the used substrate [77]. It is reported that hydrophobicity influences the cell formation, increases the number of uniform-size embryonic cells and inhibits cellular-surface attachment. On the other hand, hydrophilic substrates do not influence the cellular adhesion and consequently produce small aggregates [78]. Hydrophobicity is more emphasized here, but each cell type is unique, with cellular proliferation and growth controlled by manifold parameters.

Frequently, the substrates are bio-functionalized by means of several kind of molecules (sugars, proteins, antibodies, etc.) for stimulating specific interactions with cells [45,46,48,79,80]. Adherent cell cultures within microenvironments specifically designed to observe cells behavior at the boundaries of microchannels, have been studied and reported [12,13]. There have been attempts to make 3D cells culture in

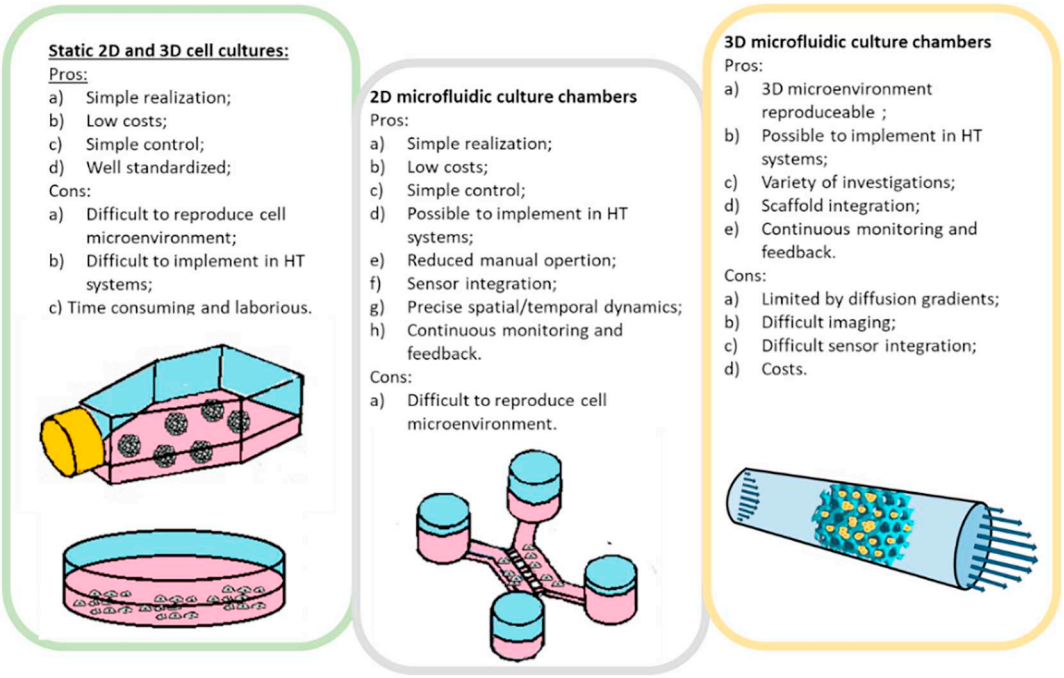

Fig. 2. Schematic representation of different cell culture models: Static 2D or 3D cell culture models, 2D microfluidic culture models, 3D microfluidic culture models.

microfluidic systems by cellular confinement in narrow micro-chambers [81]. A microfluidic platform for 3D cell cultures was developed integrating 3D scaffolds and microfluidic networks that allow controlling the fluidic environment mimicking an in vivo-like 3D microarchitecture and high-quality imaging capabilities for dynamic studies [82]. Non-adherent cells are ideally expected not to have any interaction with surfaces, but this is difficult to achieve in microfluidic devices (because of cell tethering interactions) and thereby highly variable altered behaviors could occur. It is difficult to reproduce their physiological environment, which imply that cells are subjected to certain shear stress and interactions with other cells (e.g. endothelial cells). In addition, in case of screening purposes such cells need to be trapped in specific locations, so that biofunctionalization of surfaces or traps need to be taken into account. Nevertheless, studies of non-adherent cells can be found in important application fields like, for instance, immunotherapy, where in certain protocols non-adherent cells (e.g. lymphocytes) are engineered to change their phenotype and the way they could interact with the environment and/or other cells (e.g. tumor cells). For instance, Perozziello et al. [46] exploit cell rolling in a microfluidic device to change Natural killers (NK) cells phenotype which become more aggressive against tumor cells. In general, cell rolling is a process in which the cells, injected in a biofunctionalized microfluidic chamber at a specific flow rate and shear stress, interact with the biomolecules of the chamber through the formation of specific bonds. Cell rolling is used for cell separation and isolation [83–86], physiological studies on cells [87], cell interactions [88], induction of apoptotic signals [89].

In general, it is highly demanded to probe position accuracy during the seeding process because cells motility depends also on the seeding position, for both adherent and non-adherent cells [90].

There still exists a gap in building a system suitable for culturing both adherent and non-adherent cells on a single platform due to the basic difference mentioned above, however the interactions between adherent and non-adherent cells are considered important for some physiological constructs. Hence, incorporating both types of cells in a single system will help for a careful in vitro simulation of the physiological cell microenvironment. So far, only few systems have been developed successfully for culturing both adherent and non-adherent cells on a single platform, reporting reproducible and highly reliable results [16].

3. 2D versus 3D cell cultures in microfluidic platforms

Cell culture systems are indispensable tools used in a multitude of basic and clinical in vitro research studies. There has been continuous

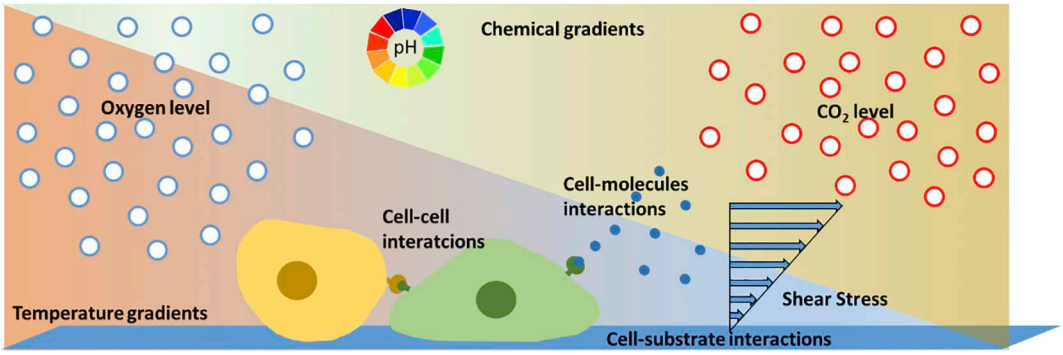

Fig. 3. Illustration showing the cell microenvironment consists of physical (shear stress), biochemical (cell interactions), and physicochemical (pH, CO_2, temperature, O_2) factors.

changes and development in the design of cell culture platforms. The classically preferred model was 2D cell culture developed almost one century ago [91]. Today several studies reveal that cells exhibit a different structural and functional behavior when seeded on flat 2D surface-coated substrate or in a polymeric 3D layer.

In a 2D cell culture system, cells grow on flat, usually plastic, dishes where they adhere and spread. Such systems have the significant advantage to allow the study of cell behavior using cheap materials and simple technologies. Moreover, the 2D cell culture systems are universally known, several protocols and extensive literature are available to scientists to analyze data and understand cell behavior in all the situations. Nevertheless 2D cell cultures have also important limits principally related to the culture environment. Since in in vivo environment all cells are surrounded by other cells and extracellular matrix (ECM), 2D cell culture does not adequately take into account the natural 3D environment. The flat surface of a 2D system does not properly reproduce the in vivo conditions of cells growth, spreading and migration, which happen in three dimensions and where each cell is in contact with another through a soft extracellular material and not on a rigid flat surface. Consequently studies regarding tissue reconstruction, drug diffusion and absorption, cancer cells expansion, and in general studies when the aim is the predictivity of a research, can give misleading results if based only on 2D systems. 2D cell culture results very often provide ambiguous and non-commensurable data with in vivo studies [92]. Development of 3D constructs is also in a fast pace because of its homeostasis compatibility (i.e. long-term stability) [74]. Additionally, the 3D environment reproduces more faithfully the ECM, the cell-cell interaction as well as the cell-ECM interaction, and consequently migration and differentiation can be driven in a 3D architecture.

Miniaturization into microfluidic platforms is very advantageous for both types of cultures. It allows the realization of multi-task systems, the inclusion of sensors for monitoring in vivo the vital parameters of cells and of ECM content, the control of pressure and temperature, a great economy in the used reagents and a high throughput, in particular for the 2D culture. Furthermore, in static systems of 2D and 3D cultures, cells metabolism and growth consume culture medium and produces waste. Consequently culture medium must be periodically replaced with fresh one to avoid dead cells due to lack of nutrients, oxygen or excess of waste substances. The introduction of microfluidic technology overcomes this problem. Nutrient and oxygen can be monitored and distributed continuously in a microfluidic culture and, at the same time, the fluid flow can bring away the toxic waste product by cell metabolism. In the case of a 3D microfluidic culture, scaffolds are inserted in the microfluidic platform and the diffusion of nutrients can be better regulated by the control of the fluid flow despite of a 3D static system, in which the lacking of an homogeneous distribution of the ECM components is a crucial drawback.

A successful liver-on-a-chip system was developed as in vitro models of human Non Alcoholic Fatty Liver Disease (NAFLD). It was designed in a sinusoid-like fashion mimicking a close tissue-like microenvironment for long-term culture of hepatic cells [93]. A myriad of interactions from other tissue cells (e.g., via signal transduction), the extracellular matrix (ECM), and other systemic factors regulate cell growth and functions, which are better considered in 3D growth [94]. There are many reports, in literature, where significant differences in the morphology, protein expression, migration, functionality, and viability of cells were revealed in 3D growth [95,96]. In spite of all advantages, with the increasing use of 3D culture techniques there are subtle technical challenges for microscopy. While 2D cultures can be conveniently analyzed by almost any kind of imaging, 3D culture constructs need special care and attention to be optimized and customized for each experiment. As a well-known fact, in vivo-like tissue-based applications are enhanced by combining 3D cell cultures and microfluidic technology, which bring to the development of integrated systems such as organ-on-a-chip and body-on-a-chip systems. The compatibility with existing high-throughput device architecture enables cost effective instrumentation, particularly important for screening approaches in 3D culture [62,97].

2D microfluidic cultures remain, actually, frequently and conveniently used to obtain results which need to be known, validate and utilized by a large scientific community. 3D systems are clearly advantageous for the possibility to realize in-vitro model very close to in-vivo organisms, but they are often very complex compared to parallel 2D microfluidic devices which are available to a larger scientific community.

4. A perspective on cell culture design – volumes

Miniaturization in microfluidic chips is characterized by small volumes of fluids introduced in the system, from microliters (μL) to nearly femtoliters (fL). Many are the benefits over conventionally sized systems. First of all the use of small volumes means less consuming of samples and reagents or nutrients in a cell culture system and consequently reduced costs. Moreover microfluidic systems can be very complex, a small platform can incorporate a lot of different functionalities and many operations can occur in the same chip. These characteristics associated with the high grade of automation that a microfluidic chip supports, consent a substantial time saving and a minor sample handling respect to a conventional cell culture, increasing the stability of the system and limiting possible external contaminations. In addition, the control of culture parameters (temperature, pressure, nutrient concentrations, etc.) becomes possible and automatable by a microfluidic chip. All these advantages widely compensate the efforts on initial designing of the system which requires to be tailored on the specific cell culture target.

Szita and colleagues [11] developed a microfabricated bioreactor, automated and remotely controlled, for the study of adherent cell culture capable with 30 nL of fluid volume. The device integrates a cell culture layer with an imaging system and a processing platform. The accuracy and precision of the image-processing data are related to the fraction of the culture area that can be imaged. The higher the fraction, the lesser the error that originates from the measurements.

Emnéus group [20] worked on development of a multifunctional platform for exploring cellular dynamics in real-time using electrochemical detection in 50 nL volume of fluid. The miniaturization also allowed the control and integration of other functions. There are also significant work done by other groups where the microfluidic systems treated volumes as small as100μL [98,99], 44 μL [54], 30 μL [100] and milliliter scale [101] that did not reduced the qualitative results produced.

5. A perspective on cell culture design – single cell culture

A microfluidic system with 1600 cell culture chambers, each of volume 4.1 nL, with integrated micro-valves for precise control and exchange of medium was reported [102]. The device was used to analyze single hematopoietic stem cell (HSC) proliferation. This low volume is particularly attractive for the analysis of rare cell types or minority subpopulations.

The geometrical characteristics such as length, width and height of microfluidic channels and culture chambers, are considered in the design, influencing in particular the flows and the shear stress of the systems. The height to width aspect ratio is given more importance in order to make the platform suitable for single cell studies, in which a monolayer culture is primarily important because suspension cells or colonies did not provide homogeneous growth and properties. Many kinds of external fields like pressure, electric, magnetic, capillary, and others affect the microfluidic flows. As dimensions shrink to lower length scales, the prominence of surface-to-volume forces increases. Proper flow values can be obtained with forces applied macroscopically, i.e. at inlets and outlets, or with forces and shear stress

locally generated inside the microchannels [79,80,103]. It is also reported that at lower microchannel height the capillary force will become the dominant source to drive microfluidic flow [104]. Although single-cell analysis plays a vital role in cell studies, understanding and application of the technique face manifold challenges: 1) Sensitivity of the different parameters to be monitored; 2) Throughput of data; 3) Easy use of the device; 4) Handling of low volumes.

6. A perspective on cell culture design – parallelization

In addition to the previous geometrical considerations, another aspect to be carefully considered in the design is the number of culture chambers or wells with the integrated sensors types. In fact, they determine the data throughput, the working efficiency, the versatility of the system, the integration of different functions and finally, the automation capabilities of the device. Most of the research groups are interested in employing 96-wells plate in order to take into account for the heterogeneity in each culture well [105,106]. This concept, pushed at its extreme, brought to the development of devices for single-cell studies [37,107]. These devices are able to control the physiological and biochemical conditions of each culture chamber separately. A precise control of cell seeding density, composition of culture medium, and feeding schedule, allows for a quantitative measurement of the cellular responses to external stimuli at each chamber, with minimal sample.

The number of culture wells covers a wide range (Fig. 4 and Table 1) and it varies from 1, e.g. for co-culture of human endothelial cells embryonic stem cell-derived pericytes [27], to 3, e.g. culture chambers for embryonic stem cells [108], to 12, e.g. cell culture chambers for exploring cellular dynamics [20], to 16, e.g. well plates for culturing of cells [109]. The number of wells has a pivotal impact over the qualitative and quantitative results produced by individual systems. A 24-well self-contained programmable microfluidic cell culture system, controlled by one flow line makes the system more efficient and robust without leakages, as reported in [100]. An alternative to expensive systems is a paper-based cell culture microfluidic system with 24 wells to study multiple assays on a single device [110], a 64-well flow-based microfluidic biochip capable of handling 64 experiments simultaneously [111].

7. Utilization of microfluidic systems in diverse applications

Applications of microfluidic devices vary from cell cultures for cell reprogramming and differentiation, bioprocesses, to cytotoxicity tests, drug testing, patch-clamp measurements and more (Fig. 5). Drug development and drug testing play an important role due to the urgency in therapies required for personalized medicines. The drug development process is highly expensive. An average time to develop and launch a new drug is 10–15 years. The purpose is to screen chemical components in a concentration-dependent way to determine chemical toxicity. Then, microfluidic platforms can also be employed in drug screening assays, which have a growing need for in vitro models to investigate better the cellular responses to chemotherapy at microenvironment level, to identify the key factors in drug resistance, and to develop drugs with better capabilities over the disease.

Drug testing is usually regulated by standard protocols and procedures, which can be divided in in-vitro and in-vivo tests before going towards clinical trials on human patients. Clinical trials is something, which cannot be changed much, but several improvement can be obtained in in-vitro and in-vivo tests by developing new tools and technologies. Conventional in-vitro tests consist in assays performed on flat and rigid substrates (agar-agar plates, Boiyden, Zigmond or Dunn chambers, etc.) where cells and test substances are put together, stored in an incubator and analyzed under a microscope. This requires manual work and specialized technicians. Sometimes, drugs are tested in combination with highly automated systems allowing to tests a huge amount of substances in parallel. However, the mentioned systems consist of expensive equipments. Miniaturized devices can be used as replacement because they would simplify the protocols, integrating more operations and innovative sensors together to pre-treat and analyze samples, reducing manual work, reagent and sample volumes and cost of analysis, ensuring at the same time high resolution, number of parallel and throughput analysis. In addition, miniaturized fluidic devices allow recreating dynamic biomimetic environments where in-vivo condition can be mimicked. This could potentially allow reducing animal in-vivo testing. On the other hand, data obtained in microfluidic devices are not always comparable with standard assays because of a different approach to treat and analyze samples and this, nowadays. is a bottleneck in using such devices for standard procedures.

In addition, microfluidic devices are employed in chemical testing. For example, on transparent mobile nematode [121], to identify drugs

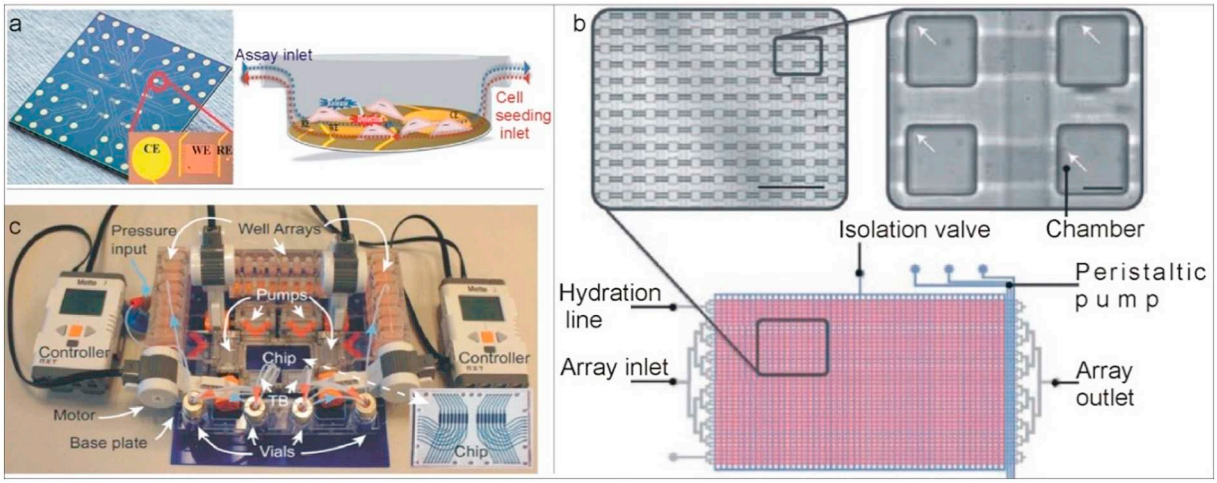

Fig. 4. (a) A multifunctional microfluidic EXCELL (EXploring CELLular Dynamics at Nanoscale) cell culture and analysis platform. A photograph of a 12-microelectrode array chip (insert: zoom on one measurement site with 3-electrode configuration (counter (CE), working (WE) and reference electrode (RE)). In the right, a schematic representation of an individual cell culture chamber [20] (b) An isoosmotic perfusion microfluidic cell culture array. Scheme of the device with micrographs as insets. The cell culture layer contains 1600 chambers (pink) connected by flow channels (gray). The control lines (blue) consist of an isolation valve and a peristaltic pump to control cell loading and perfusion rates. Arrows point at single cells. Scale bars, 1 mm (left) and 100 μm (right). [102]; c) A portable MainSTREAM system with four micropumps and an integrated reaction chip with up to 32 cell culturing chambers. [109].

Table 1
Examples of microfluidic devices used for specific applications at different parallelization, automation and sensor integration levels.

Application		Parallelization (chambers or wells)	Automation	Sensor integration
Cell culture	a. Cancer Cells	384 [15], 96 [99,112–114] 64 [111], 24 [100,110,115], 16 [109], 12 [20,22], 1 [11,27,116]	Data acquisition Fluid handling Environmental control	Pressure sensor pH sensor Thermal sensor
	b. Stem Cells	96 [105,106], 75 [28], 10 [31], 6 [37], 3 [30], 1 [27,29,101]	Data acquisition Fluid handling Environmental control	Thermal sensor pH sensor Pressure sensor
	c. Organ-on-chip	6 [55,117], 4 [62], 2 [56], 1 [53,54,57–59,118]	Data acquisition Environmental control Fluid handling	Fiber Optic sensors (O_2) Temperature sensor & controller pH, DO, CO_2 sensor
	d. Body-on-chip	2 [65], 1 [64]	Data acquisition Fluid handling	Optical sensors
	e. other cells	96 [98], 6 [119], 3 [33], 2 [14]	Data acquisition fluid Handling Environmental control	Pressure sensor pH sensor Thermal sensor
Drug screening		32 [120] 8 [121], 4 [122,123], 5 [124], 7 [115], 2 [118], 1 [125–127]	Data acquisition fluid Handling, motion tracking system, Image acquisition	–
Cryopreservation of cells		96 [69], 1 [70]	Data analysis	–

Fluid handling – Automated liquid handling system, Actuation of valves. Ambience/Environmental control – pH, temperature, oxygen, pressure, osmolality monitoring. Data Acquisition & Analysis – Image acquisition and data analysis, Statistical data analysis. Fibroblast production & differentiation – Fibroblast production, Reprogramming, iPSC purification, Parallel culture of iPSCs, Analysis of differentiation propensity, Automated differentiation.

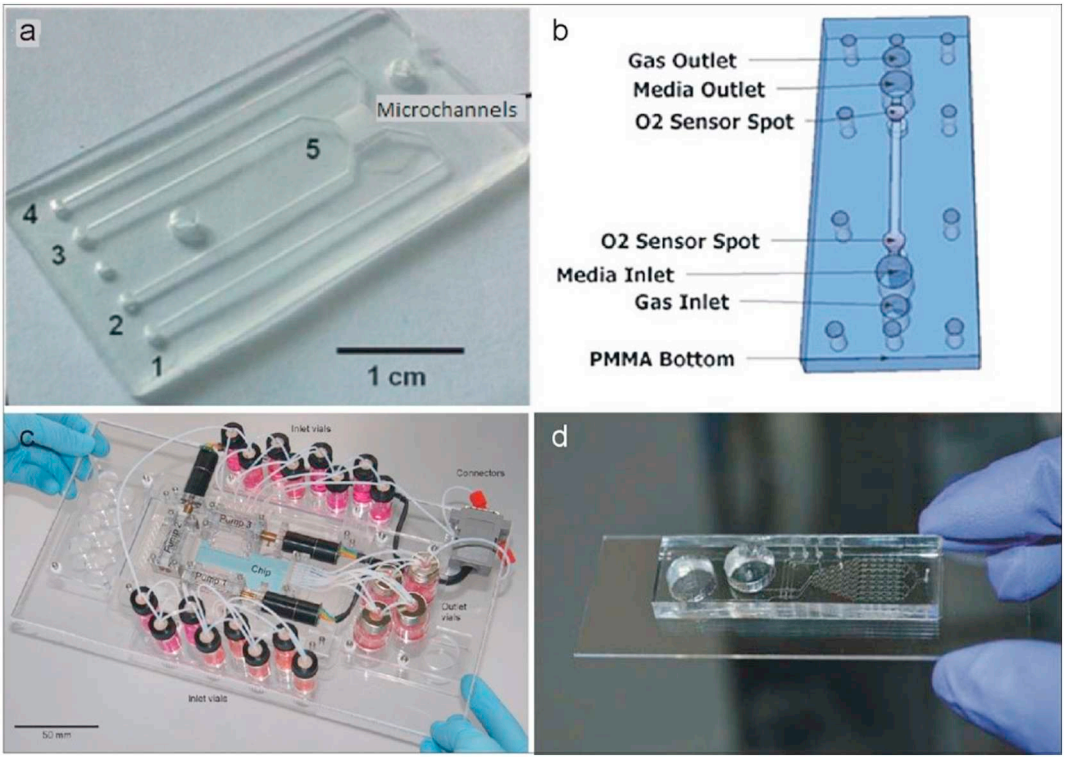

Fig. 5. (a) Microfluidic device where 1- inlet for upper chamber, 2 – inlet of bottom chamber, 3 – outlet of bottom chamber, 4 – outlet of upper chamber, 5 – filtering membrane [47] (b) Schematic of Microbioreactor for long-term cell culture in controlled O_2 environment [118] c) Experimental set up of a microfluidic cell culture system with 16 inlet vials and 4 outlet vials [100] d) Microfluidic high-throughput device for cytotoxicity screening test [120].

that block dispersal of a highly invasive bladder carcinoma [126], further, to study hypoxia-dependent cytotoxicity of anticancer drugs by generating oxygen gradient in a microfluidic device [128]. These are employed for parallel toxicology testing [123] to probe anticancer drugs on uniform sized spheroids analyzed with flow cytometry, and to understand the drug mechanism in 3D cell culture [127]. Further significant examples can be found in [60,120,122,124,125,129]. Screening of chemo-sensitizing compounds and cytotoxicity with reduced adverse effects are promising for clinical usage.

Besides drug testing and cytotoxicity screening tests, microfluidic devices with several designs, dimensions and integration to other external equipment, are used in other biomedical applications, like cryopreservation of cells [69] where the excessively passaged cells are preserved in microfluidic devices for easy handling and quick availability for further processes.

Tissue engineering is another upcoming field of regenerative medicine, which mainly deals with “off-the-shelf” bio-artificial organs, and regeneration of injured tissue in a living organism [130]. In tissue engineered culture, extensive cultures are required to create functional tissues and biological structures in vitro: survival, growth and

inducement of functionality should be ensured [54,66,68].

Detailed study on cell-cell interactions are important for several aspects of cell life and development. These interactions allow cells to communicate with each other in response to changes in their microenvironment. Thanks to microfluidics laws, microenvironment is mimicked by microfluidic devices with stromal-epithelial cells [22] and tumor-stroma interactions [113]. Identifying how the microenvironment regulates stem cell activity is central to understanding stem cell biology and to develop strategies for therapeutic manipulation of stem cells. This is motivated by the monitoring of essential parameters like oxygen, because hypoxic conditions are known for rigorous metastasis condition in tumor patients. Thus, multiple systems, with remote automated measurements are developed by various research groups worldwide [118,131–134]. Similarly, deregulated pH inside cells and in the cellular microenvironment are well-known emerging features of most forms of cancers. In addition, cellular acidity is a highly efficient mechanism of drug resistance, hence the close monitoring of pH is also accomplished by microfluidic devices for enhanced analysis of cell microenvironment [135]. Microfluidics can be used in combination with patch-clamp techniques to enhance methodology of measurements on cells [136,137].

Extending to the aforementioned applications, a significant use of microfluidic systems is cell reprogramming, iPSC culture and purification [30,108]. Recently, a particular interest quickly growing is the monitoring of stem cells differentiation through microfluidic systems. In fact, the fate of stem cells is greatly controlled by microenvironment that promotes stem cell maintenance and controls the differentiation to achieve homeostasis. The main reasons to employ microfluidic systems in such critical studies are: a) high-throughput screening of a wide range of physiological, biological and biochemical parameters, and b) customization of the physiological environment to heterogeneous and 3D growth conditions [138]. In addition, cells are studied at their natural state with a high timing control, which is a crucial issue for primary adherent cells and cells derived by differentiation from stem cells, e.g. neurons [139]. A microfluidic system integrated with “iso-osmotic bath” for medium preservation is used to study single hematopoietic stem cell (HSC) proliferation. This device is predominantly attractive for the analysis of rare cell types or minority subpopulations [102].

In-vivo cell populations change dynamically, therefore it is complicate to interpret data. For gene expression within its appropriate biological context, it is necessary to distinguish differential gene transcription from gene expression induced by changes in the cell populations. In such cases, an independent, cell-specific gene expression data set is created to assess and to identify the appropriate cell type, and to provide insight on the optimal enrichment level that minimizes the number of false discoveries. This indicates that analysis of distinct cell subpopulations may yield more clear-cut results than analysis of non-separated data [44,49,80,140]. Moreover, important insights are obtained when investigating the alteration of protein expression and in vitro chemo-sensitivity of cells [14,33,141].

8. Automation – a benchmark parameter in current microfluidic systems

The major impacts in the literature of microfluidic systems are tagged with the level of platform automation. In order to automate a platform, researchers work at different points that can be regarded as automation nodes of the system. The steps range from having automated essential cell culturing devices [11,119], to cellular seeding, preparing, storing, passaging and harvesting [105,142], to image acquisition and cell analysis [112,143–145], up to automated liquid handling and sensor systems [15,16,28,100]. A system developed with high automation capability for fibroblast production, reprogramming capability, iPSC purification coupled with parallel culture of iPSCs, followed by automated analysis of differentiation propensity has been reported [106] with notable advantages, such as: 1) Five- to six-fold reduction in reagent cost; 2) Ten- to twelve-fold increase in productivity; 3) High-throughput due to full automation.

In current literature, image acquisition and analysis is a commonly automated trait [146,147]. Time-lapse imaging [27,108] is commercially available with open source software like ImageJ, Image-Pro Plus (IPP), XEI software, Imaris, NIS ElementsD, Olympus CellˆD, Meta Morph, Analyst, LabChart, R, V3D-Viewer and Matlab scripts. These programs have highly extensible features, accompanied with plugins, like homemade macros and scripts that aids the customization according to the research needs. A high sensitivity (~0.94), high precision (~0.96), high accuracy (~0.97) when using PHANTAST software toolbox (built with Matlab and ImageJ) has 3.6-fold increase in precision over human estimation besides being a robust and convenient tool to generate qualitative and quantitative data without the need for detachment of cells [11]. In addition, microscopic and macroscopic image acquisition systems are also employed for accuracy and precision data [57,117,127].

Robotic liquid handling systems are also gaining attention combined with the specialized needs, like medium exchange [11], fluidic activation of syringe pumps [100] or miniaturized infusion pumps [148] connected with reservoirs and wells plates. Other operations as transportation of soluble factors based on custom-made programmable schedules [111], liquid switching between containers [16,108], setting of flow rates [15,135], and pressure generation with perfusion rates [34,119] are available. Lately, a multitude of ready-to-use automated syringe pumps are commercially available, like Cetoni, Harvard, kDScientific, that provides very useful customizations for research groups, such as pneumatic automated controller system to operate micro-valves aids in easy handling [120]. Moreover, automated liquid handling system is adapted in many platforms to enable high-throughput, large-scale production assays and removal of manual interference for a fully controlled and undisturbed microenvironment which ensures high reproducibility of results. Thereby, it can be predicted that automation is extensive without any limitation on any particular component or characteristic, but it purely depends on the requirements according to which different processes or analytical procedure are automated.

9. Integration – a promise towards high-throughput platform

Cell culture in microfluidic systems is gaining increasing attention for three predominant attributes of platforms: (a) Control of microenvironment (b) High-Throughput systems and (c) Integrated functionality. Along with these advantages cell culture in these systems also poses challenges and critical bottlenecks that need to be resolved. In most cases, cells are enclosed in a microenvironment that hardly communicates with the outside world, thereby limiting the integration of monitoring systems from macro-scale to micro-scale. In addition, very low volumes of samples are involved in such systems that restrict the use of existing laboratory techniques, demanding high volumes analysis to benchtop systems as for example for pH monitoring [135]. Development of microfabricated systems are expanding swiftly, nevertheless simple, feasible and quick techniques adaptable universally are falling short. Understanding the demand for integration of multiple sensor systems, controllers, liquid handling systems and downstream assays allows for measurements performed at controlled microenvironment. The study of cells includes structural and functional occurrence, organization of organelles, their physiological properties, metabolic processes, signaling pathways, life cycle, and interactions with their environment. There are quite interesting innovative systems that need special mention for their reasonable and sensitive results: studies on *X*-ray analysis [149], scaffold-based systems for target discovery [52,150,151], UV/Visible transparent optical waveguides fabricated using organic and inorganic nanocomposite layers [152], devices with optimal optical properties [153] and multiple microarray

microsystem [154].

A stand-alone customizable single use, disposable cartridge manufactured in high volumes at low costs to service multiplexed and multiclass testing in various biomarker detection is a powerful POC (point-of-care) tool, aimed at developing a fully integrated device with major considerations on waste containment, low contamination and sensor integration [155]. The programmable Bio-Nano-Chip (pBNC) and its exploded view of internal circuitry is shown in Fig. 5 and illustrates the miniaturization and integration efficacy at its best. Extended use of microfluidic devices in micronutrient analysis with electronics enabled paper-based analytical devices is an appropriate example for a fully integrated system with little or no manual interruption in low resource settings [156]. A microscale perfusion 3-D cell culture platform that handles an integrated system for thermal control, multi-channel medium pumping, 3-D cell culture sample loading and detection techniques has proven to be more user-friendly for biologists [15]. Integration for electric control and infusion flow pump for flow rate control, in the range of a few μl/h, resulted in cell proliferation rates comparable to conventional methods [148].

Apart from laboratory based microfluidic systems, various diagnostics industries have launched their patented and customizable microbioreactors, e.g. Ambr™, which is an integrated system for cell culture with closed loop control of pH and DO (dissolved oxygen) sensor, automated liquid handling for reactor set-up, automated feeding, base addition, sampling, and optional integrated cell viability analysis (Vi-CELL®) [157]. Microfabrication plays a vital role in lab-on-chip and organ-on-chip systems, where fabrication of micro-components like pumps, valves etc. need high levels of accuracy [64,120]. A hydraulic driven micro valve and a peristaltic micropump have been integrated in a body-on-chip for drug screening [64]. This is an efficient solution in terms of interfacing and integration, since multiple tubings and connections outside the platform have been eliminated. *Min-Hsien Wu and colleagues* built a system (Fig. 6a) with pneumatic control module and thermal control module for continuous nutrient supply, waste removal, in schedule flexibility, liquid handling systems coupled with high automation capability, featuring 30 microbioreactors on a single platform. This system is considered a microscale, high-throughput, perfusion, 3D microfluidic cell culture system with high success rates [15]. A lab-on-a-chip device (Fig. 6b), with a handling platform made of metal or plastic frame, where the microfluidic devices are placed and clamped, was designed by *Mayr and team* [57]. Fluidic interfaces [158–160] were directly integrated into the platform to connect external valves and pumps through tubes without actually touching the device, and through an optical window for fluorescence microscopy and live cell imaging. Finally, a heating element is also integrated into the device to control the temperature at the desired level, along with a luminescent oxygen sensor (made of highly photo stable benzoporphyrin dyes) to perform stable oxygen sensing and connected to a commercial oxygen meter read-out [57].

In order to quantify the working efficiency of a microfluidic system, it is important to specify the type of sensors interfaced to the system and the number of parameters measured throughout the analysis. When a system is set to observe the metabolism and proliferation of cancer or stem cells, it is always necessary to have a steady check on other factors resulting from their activities. Hence, it is a mandatory design rule to consider the necessary sensors to be included in the system [52]. For example, the control and monitoring of pH is crucial to ensure a certain cell viability rate. An innovative design with single-pass light absorption measurement and simple imaging technique is developed for pH measurement with volumes of the samples in the range of nL, even at the absorption level as small as 1% [135]. There are inventive and unique designs available in literature to monitor oxygen and pH either on-chip or off-chip, but an obvious challenge is the fabrication complexity [116,161–163]. *Li* et al. observed the cell behaviors at different pressure levels where the system was composed by a syringe pump, for medium perfusion and pressure generation, and a digital manometer to monitor pressure [164] and other pressure sensing activities [54]. Similarly, temperature plays a vital role in cell growth and vitality [112,119,165]. Moreover, on-chip electrodes can be included to measure electrochemical activities in neuronal cells [166].

Oxygen is considered one of the most important factor that characterizes the cellular activities. Hence, preference is given on stable and finite measurement of oxygen in the cellular microenvironment. Remote oxygen monitoring system with 0.03 standard deviation accuracy is reported in the literature [131]. With the advent of multiple commercial sensors, the most prevalent ones are the optical sensing elements due to their compatibility with micro-world and realization of different architectures, such as luminescent dyes, sensor beads or sensor layers combined with extendable read-out methods [132].

Temperature also is a key parameter in cell studies. Due to the lower time constant, microfluidics has strong potential for applications involving temperature changes faster than 1 min. In most cases, temperature needs to be maintained at 37 °C but there are also instances that demand different temperature conditions, depending on the type of investigation and analysis involved. When the cells are passaged and need to be preserved, microfluidic systems can adapt to the cryopreserving temperature [69]. A study also revealed the effects of increased temperatures in cell-adhesion [167]. Hence, constant monitoring and reliable temperature of the system become crucial points to investigate on both physiological and morphological entities of the cells. Nearly in all cases, an accurate temperature control is needed in order to preserve the cells alive and for feasibility of their proliferation at a considerable rate.

Several sensors are available for measuring various parameters of the analyte, also exploiting plasmonics [168,169] as described in the following sections.

10. Optical imaging in highly integrated microfluidic devices

With the development of Microfluidic Devices in biomedical research, the complexity of their structures increased significantly, especially in the recently developed highly integrated organ-on-chip and human/body-on-chip systems [170]. Optical microscopy imaging is one of the most popular and reliable analytical techniques in microfluidics applied to biomedical issues, and many outstanding works have reported results with and without fluorescent labeling [116,168,171–173]. However, the practical applications are limited in such heterogeneous 3D not transparent organ-on-chip and human/body-on-chip devices, through which optical imaging is difficult.

As a smart compromise, assembling laminated Microfluidic Devices design was proposed [174]. Briefly, various cell-lines were cultured in a battery of single layer microfluidic chip, and these layers were laminated and integrated as a functional mimic organ. Although this laminated microfluidic devices could be imaged layer-by-layer with universal optical microscopy after disassembling, the real-time functional imaging of inside cells and/or large volume tissues are limited. However optofluidics, i.e. a high integration of optical imaging and microfluidic systems, has been developed [168,175] also embedding specialized elements in microfluidic platforms, such as plasmonic nanodimers [172] and optical tweezers [41], and extremely high quality signals have been achieved. Among these optofluidics devices, the light-sheet imaging based designs are distinctive by integrating light-sheet illumination into microfluidics. Unlike the general wide field and confocal optical microscopy, the light-sheet microscopy (LSM) or selective plane illumination microscopy (SPIM) has inherent advantages for real-time large-scale biological specimen imaging, due to low photo-toxicity and capabilities for three-dimensional imaging of tissues and organs [176]. Not too long ago, the researchers had shown the two different minds, i.e. embedded fiber-lens light-sheet illumination structure and nanofabrication based self-reflective light-sheet illumination (Fig. 7), to build optofluidic devices by integrating the LSM with microfluidic devices for imaging of millimeter scale biological specimen in highly

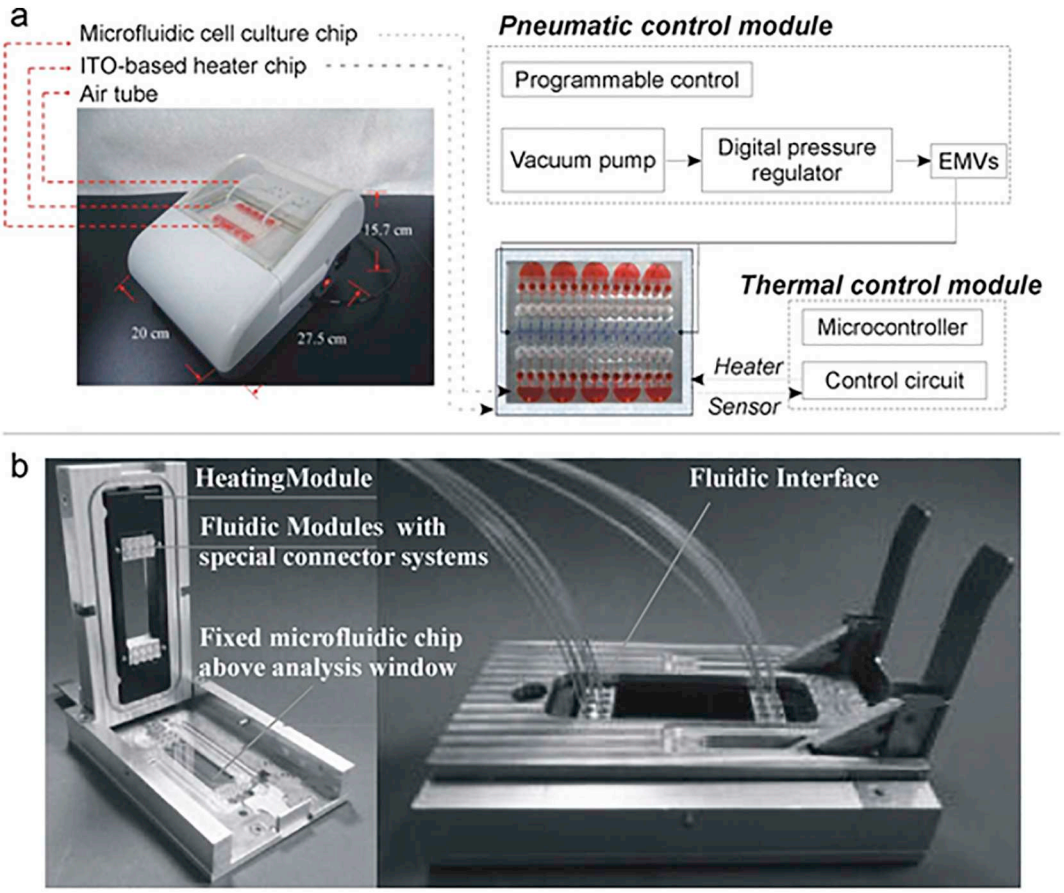

Fig. 6. (a) Schematic representation of an automated and integrated 3D cell culture microfluidic system and overall experimental set up [15]. (b) Lab-on-a-Chip interface platform [57].

integrated platforms [177,178]. Certainly, these optofluidic devices based methods are elegant and ingenious, but they also reveal a critical lack of universality. At this regard, a universal LSM has been built to image the 3D real-time convection in droplet deposited over super-hydrophobic surfaces: by means of high-speed camera, the millimeter-size scale specimen could be imaged up to 200 frames-per-second. Compared to other optofluidic devices, this LSM shows highly universality and versatility, and it constitutes a promising candidate as a next-generation tool for microfluidic devices dedicated to imaging and biomedical studies involving both in vitro and in vivo crystallization of biomolecules.

11. Raman spectroscopy and advanced systems of optical trapping in highly integrated microfluidic devices

The development of microfluidic devices and the possibility to integrate specific elements in microfluidics is a great improvement in cells or biological elements analysis. The assembling of filters, micro-traps and sensors inside a microchip allows separating and driving single cells or particular biological substances, in diverse areas of a lab-on-chip, where different stimuli can be applied and the responses are monitored.

In this regard, metallic nanodimers integrated in a microfluidic device represent an optimal instrument to analyze a single cell at sub-cellular level, obtaining a preliminary Raman-based flow cytometry (Fig. 8) [172]. Metallic nanodimers are powerful optical sensors, which enhance the results of vibrational spectroscopy once they are coupled and probed with an external Raman spectrometer, thus allowing for fast and multiparametric analysis of single cells. In other word, they behave as SERS (surface enhanced Raman scattering) devices, generating a local electromagnetic field enhancing Raman scattering, and consequently increasing the sensitivity of the device [179]. The microfluidic platform integrating nanodimers consists of five through-channels comprising microfluidic traps, simple volumetric constrictions of the channels, where cells can be temporarily captured to record their Raman spectra. The movement of each cell into the trap allows the analysis of a specific part of the cell. The resultant spectroscopic information comprehends not only the whole cell signal, as from a standard Raman analysis, but also the enhanced signal coming from both the cellular membrane and the cytoplasm.

Optical Twizer trap. Left: (A) Isometric representation of the optical tweezers trapping a particle and (B) optical image of a trapped red blood cell. Right: Raman spectrum of a colon cancer cell in the C—H stretch Raman band [41].

Optical traps can also be hosted inside a microfluidic device with a huge advantage for fluorescence and/or Raman measurements of single cells. They can be obtained by integrating in the devices optical tweezers (OT), realized through tightly focused free-space laser beams or by counter-propagating beams (Fig. 8). An alternative and valid solution to make an optical trap by counter-propagating beams consists of a miniaturized fiber-based OT [41]. The beam shaping required to create the optical trap is achieved by microprism reflectors fabricated by Two Photon Lithography (TPL) on the fiber facets. Several micro optical traps, fabricated in this way, can be integrated into different sections of a single microfluidic chip, making possible a different optical stimulus and then a different kind of analysis (e.g. Raman spectroscopy or fluorescence microscopy) of the single cell in each section of the chip. This trapping system ensures flexibility and possibility of complete integration in a lab-on-chip, guarantees cell-safety, allows for treatment and analysis of single living cells in a laboratory under the microscope.

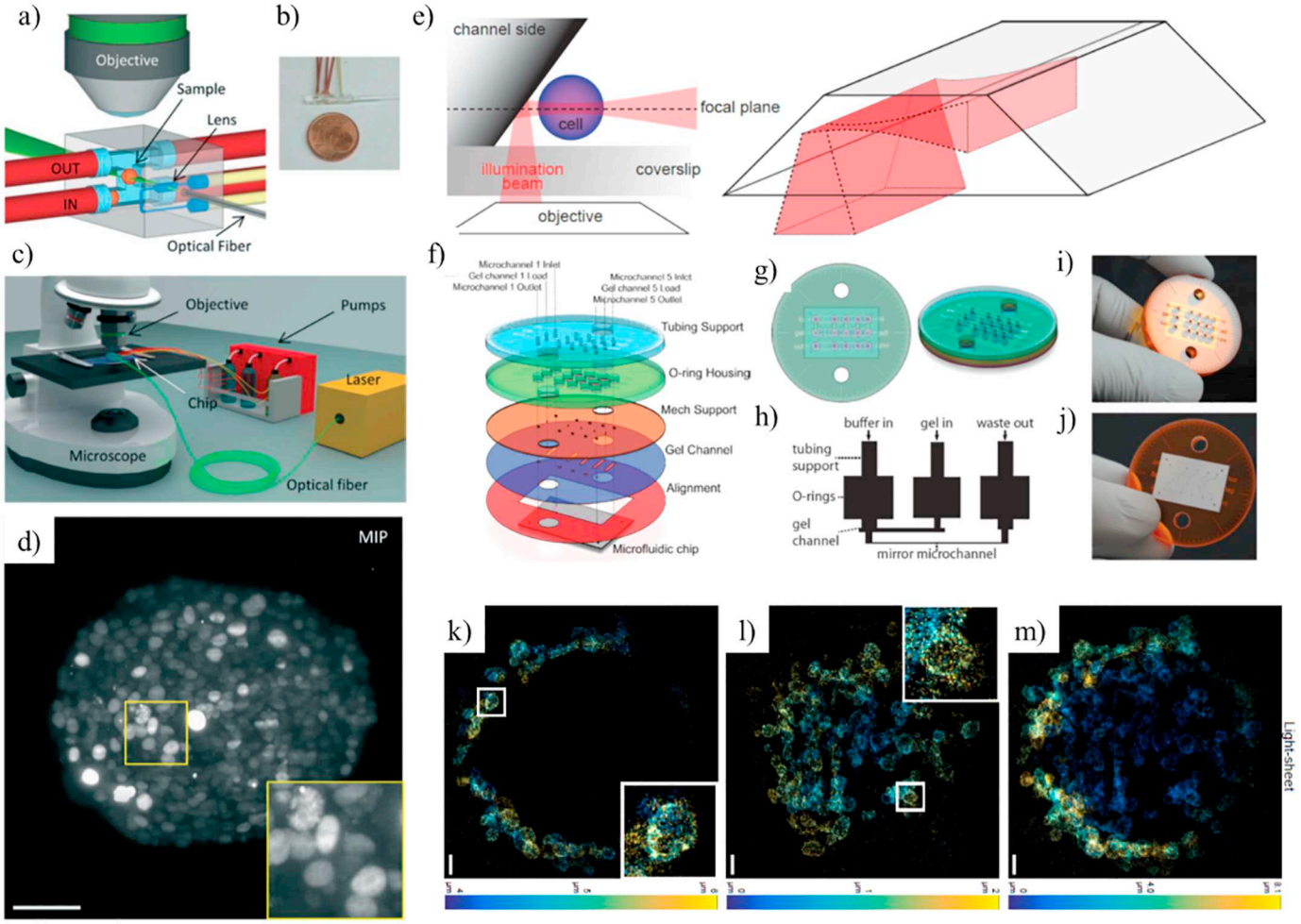

Fig. 7. (a) Scheme of the embedded fiber-lens light-sheet illumination system; (b) Picture of the chip with 1 € cent coin for size comparison. (c) Scheme of the imaging setup: the chip on a microscope stage and the sample collected from a reservoir and delivered to the chip by a pumping system connected with the red tubes. (d) Automatic imaging of cellular spheroids. [177] (e) Principle of reflected beam light-sheet microscopy. A laser line is focused through the objective and is reflected from a 45° mirror sidewall of a microfluidic channel. The light sheet is reflected at the focal plane of the objective, thereby illuminating only the in focus plane of the cell. 3D optical sectioning is achieved by moving the channel and cell up and downwards and repositioning of the light sheet such that it is reflected at the focal plane. (f) Exploded view of chip packaging. Each chip is packaged into five layers of PMMA with two O-rings incorporated for each in and outlet. (g) Transparent top and side view images of chip assembled in packaging. (h) Schematic showing the fluidic connections inside the chip. (i,j) Photographs showing top (h) with tubing connections and bottom (j) with coverslip surface of packaged chip. x-y projections of reconstructed images with color coded z-depth are shown for cells imaged using light-sheet illumination (k,l,m) [178].

12. Conclusions

The microfluidic field has evolved to be an integral part of cell biology studies, since the implementation of this technology has left no stones unturned, starting from cell culture, drug screening, drug discovery, tissue engineering to patch-clamp measurements. In addition, by virtue of the unique properties and versatile features, microfluidics holds great promises for numerous applications in chemistry, engineering, biology, biomedicine, and other fields.

In general, microfluidic devices give several advantages for cell biology applications. Some advantages come from the fast response of the microdevices, low reagent consumption (nL), the opportunity to manipulate large number of cells simultaneously and independently, automatic generation of a large number of different individual conditions, and easy integration of numerous analytical standard operation and large-scale integration. Finally, the versatility of these devices partly enables the simulation of in-vivo cellular microenvironment (vascularization, 3D, nutrient stress, etc.…).

However, microfluidics have also some drawbacks. Since laminar flow dominates in microfluidic devices, the only mechanism allowing for mixing relies on diffusion, which is a very slow process. This is a big limitation for certain applications, e.g. where chemical gradients are strongly undesired. In addition, changes in scaling can further give difficulties in adaptation of biological protocols to fit experiments in microsystem (i.e., media and cell concentrations). These differences require careful comparison between data obtained in macroscopic experiments and data obtained in microsystems. Furthermore, microfluidic devices need often complicate set-ups to be handled and managed. Consequently, even in presence of inexpensive disposable microfluidic devices, the costs are related to the instruments, which are needed to run the microfluidic devices or the integrated sensors. Moreover, complex instruments require specialized personnel and, in some applications, they are often inversely proportional to the throughput of the produced data.

In conclusion, microfluidic devices emerged a century ago but their development until date has reached no saturation due to diverse and distinct requisites needed for real lab practice. Notably, a continuous and constant curve in development and optimization of fabrication methods, discovery and invention of biocompatible materials, self-contained and competent microfabrication of sensors, powerful and

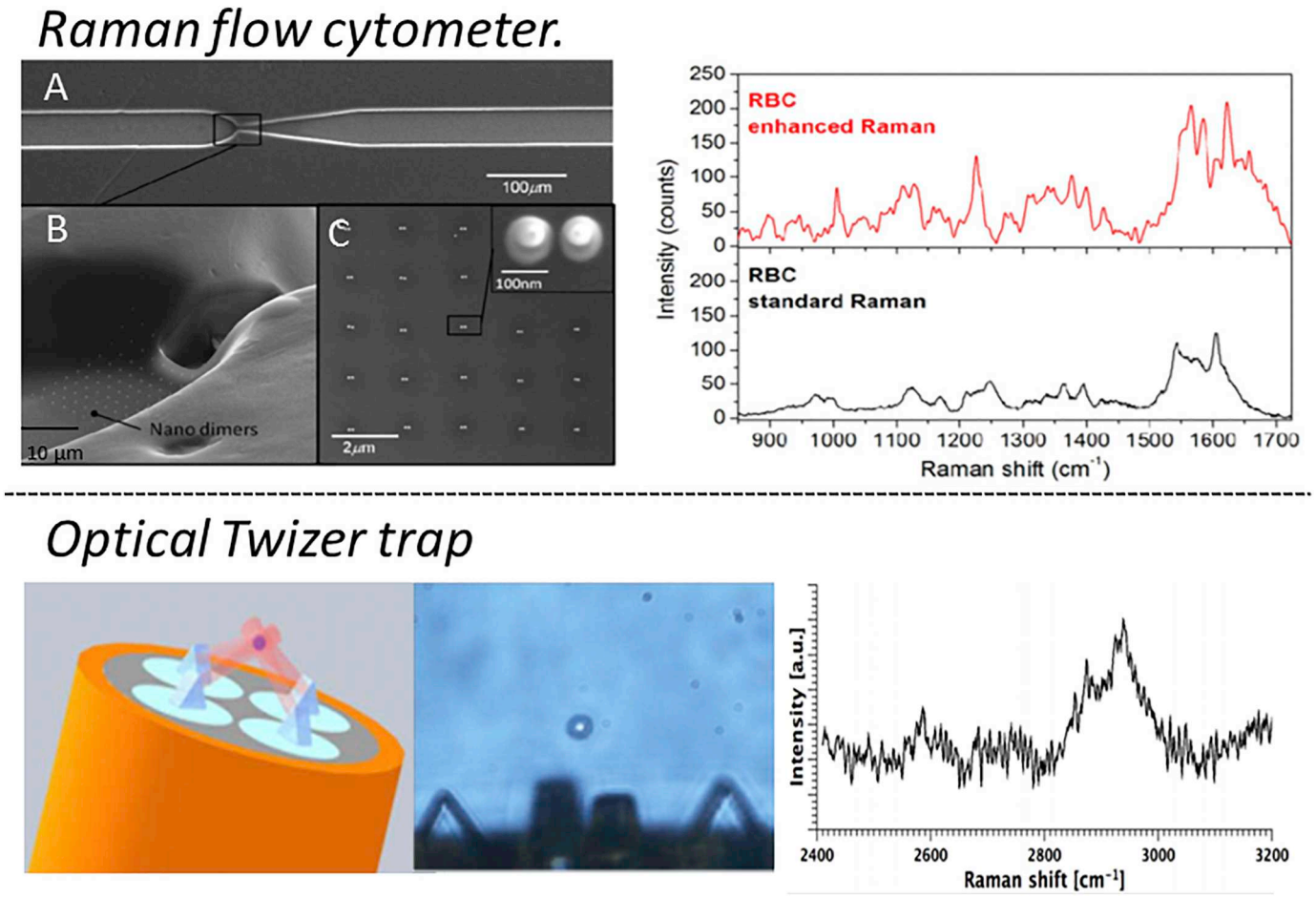

Fig. 8. Raman flow cytometer. Left: Scanning electron image of the microfluidic trap and the integrated array of nanodimers. (A): Top view; (B): isometric view; (C): zoom of the integrated nanodimers. Right: Raman spectra collected from red blood cells (RBC) on nanodimers (used power 10% of the total) and on a flat substrate (used power 100% of the total) [168].

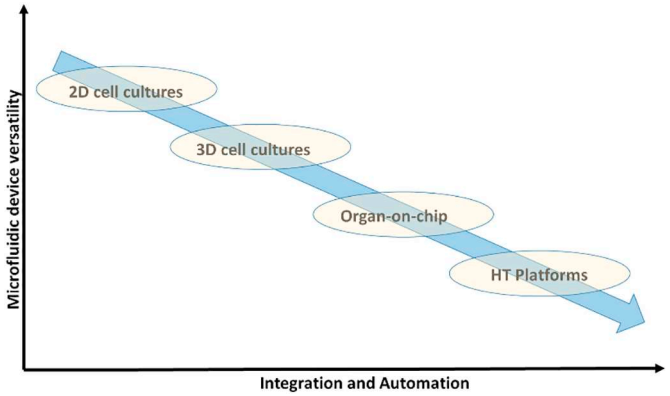

Fig. 9. With the increasing level of integration and automation, the Microfluidic Devices lose their versatility due to several constrains and compatibility of the operation integrated and automated against a general application cumbersome flow dynamics.

productive integration of sensors, adept and dynamic automation of intricate analysis is acclaimed. The exclusive properties of the device that involves multifunctions on a fully dynamical control of the bio-environment make microfluidic devices attractive in cell biology studies. Microfluidic systems are compliant with other techniques. Biochips can be fabricated with multiple microchannels integrating ultrasensitive detecting systems, which can be employed in cell analysis and cell manipulation studies at high temporal and spatial resolution. Microfluidics can be applied for manifold analyses that result in high throughput and high sensitivity diagnosis. In single cell studies, cell heterogeneity is the ruling factor that needs exceptional attention; microfluidics has made it possible with the establishment of controlled cellular microenvironment resulting in determining the behavior and response of single cells towards variations in the surrounding micro-environment. During this review, we did an extensive excursus on the degree of integration, automation, parallelization and high-throughput of data against versatility of microfluidic device for cell cultures (Fig. 9). By increasing the number of samples (number of culture wells) and sensors in a single device, multiple unique parallel analyses and comparative studies can be performed on a single experiment. On the other hand, when the number of parallel samples increases, the device handling becomes more difficult; risk of contamination should be taken in account and time consumption is higher for completion of a single analytic cycle. However, there is a high risk of inconsistency in expression and heterogeneity of cultured cells when the number of samples analyzed in a single experiment is decreased. Versatility decreases as the level of integration and automation increases due to specific parameters monitored and analysis performed in parallel constrained by a specific application. Automation capability is more complicated to be handled for parallel activities, thus high-throughput is compromised, and it is inevitable to observe an increase of production costs.

The diagnostic industry is also constantly investing in research and development of such equipment, which would bring high standards in their respective sectors. The future development in microfluidic devices will mainly focus on fabrication methods, on biomaterials used in

fabrication as an alternative to the existing PDMS, PMMA, PS, PC or glass. Monitoring systems (interface of multiple sensors into one single system) would result in a portable microfluidic device that becomes a fundamental tool in diagnostics. In countries with low resources, poor national health systems, considerable chances of contagious diseases are reported that brings massive rates of mortality and morbidity. Hence, disposable microfluidic lab on a chip could be of strong interest and cost affordable device for both diagnosis and medication in order to improve the health level. A fully automated, highly controllable, integrated system is expected to address the pressing shortcomings of today cell biology studies. Besides the rapid, low-cost and automated analytical procedures, synergy sought from cell biologists, pharmacologists, toxicologists, biostatisticians and microfluidic engineers will yield massive revolution in the field that needs to be addressed at the earliest. With the mass production of microfluidic analysis systems, portable and inexpensive microfluidic devices for POC diagnosis are promising in the next decade with the new inventions that certainly will enter the global market.

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