Advanced technological tools to study multidrug resistance in cancer

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ABSTRACT

The complexity of cancer biology and its clinical manifestation are driven by genetic, epigenetic, transcriptomic, proteomic and metabolomic alterations, supported by genomic instability as well as by environmental conditions and lifestyle factors. Although novel therapeutic modalities are being introduced, efficacious cancer therapy is not achieved due to the frequent emergence of distinct mechanisms multidrug resistance (MDR). Advanced technologies with the potential to identify and characterize cancer MDR could aid in selecting the most efficacious therapeutic regimens and prevent inappropriate treatments of cancer patients. Herein, we aim to present technological tools that will enhance our ability to surmount drug resistance in cancer in the upcoming decade. Some of these tools are already in practice such as next-generation sequencing. Identification of genes and different types of RNAs contributing to the MDR phenotype, as well as their molecular targets, are of paramount importance for the development of new therapeutic strategies aimed to enhance drug response in resistant tumors. Other techniques known for many decades are in the process of adaptation and improvement to study cancer cells' characteristics and biological behavior including atomic force microscopy (AFM) and live-cell imaging. AFM can monitor in real-time single molecules or molecular complexes as well as structural alterations occurring in cancer cells induced upon treatment with various antitumor agents. Cell tracking methodologies and software tools recently progressed towards quantitative analysis of the spatio-temporal dynamics of heterogeneous cancer cell populations and enabled direct monitoring of cells and their descendants in 3D cultures. Besides, novel 3D systems with the advanced mimicking of the *in vivo* tumor microenvironment are applicable to study different cancer biology phenotypes, particularly drug-resistant and aggressive ones. They are also suitable for investigating new anticancer treatment modalities. The ultimate goal of using phenotype-driven 3D cultures for the investigation of patient biopsies as the most appropriate *in vivo* mimicking model, can be achieved in the near future.

Keywords: Cancer multidrug resistance; New generation sequencing; Atomic force microscopy; Single live-cell tracking; 3D cultures; Microfluidic devices

Introduction

Cancers comprise various cell populations with distinct phenotypic and genotypic profiles as well as with an inherent potential for metastasis (Marusyk and Polyak, 2010). A recent definition describes cancers as epigenetic disorders where cells emerge and compete under a robust evolutionary pressure (Vitale et al., 2019). The extracellular environment can produce a positive selection of subsets of pre-malignant cells with a fitness advantage towards metastasis and colonization of healthy tissues (Martincorena et al., 2017; Ostrow et al., 2014). Heterogeneity leads to the expansion of diverse niches, including hypoxic or perivascular regions that might support the development of cancer stem cell phenotypes and drug-resistant cell populations (Fu et al., 2015; Junttila and de Sauvage, 2013; Mao et al., 2013; McGranahan and Swanton, 2017; Tellez-Gabriel et al., 2016).

Cancer multidrug resistance (MDR) has been a subject of intensive research in the past four decades in an attempt to decipher the molecular mechanisms underlying MDR and develop novel modalities to surmount this major therapeutic impediment (Cui et al., 2018; W. Li et al., 2016; Livney and Assaraf, 2013; Robey et al., 2018; Zhitomirsky and Assaraf, 2016). Still, the implementation of the significant amount of the generated findings in this field into clinical practice is rather insufficient. New emerging tools are necessary to address many burning questions regarding cancer MDR. Characterization of different MDR phenotypes (Assaraf and Borgnia, 1994; Goler-Baron et al., 2012; Goler-Baron and Assaraf, 2011; Ifergan et al., 2005; Zhitomirsky and Assaraf, 2017, 2015), correlation of their presence with cancer aggressive behavior and identification of new druggable targets to overcome MDR, are some of many important tasks in studying cancer MDR.

Cancer is a complex disease that systematically affects the whole organism. Characteristics of cancer cells and their tumor microenvironment (TME) favor the progression of the disease and invasion of remote organs, distinct from the primary location of the cancer. These characteristics or hallmarks of cancer have been described and revised by Hanahan and Weinberg (Hanahan and Weinberg, 2011, 2000). In its latest revision, 10 characteristics of cancer were identified, the study the of which is hardly possible with classical two-dimensional (2D) cell culture methodology.

Cell-culture based screening of anticancer drug effects has significantly evolved in the past decades. For a long time, conventional 2D cell cultures have been employed as the sole *in vitro* model to test the anticancer activity of new drugs. In this respect, the NCI-60 human tumor cell lines screen using a panel of 60 human tumor cell lines of distinct cell lineage was introduced by the National Cancer Institute in Bethesda, MD, USA. These cytotoxicity studies on cells grown under monolayer conditions are quite affordable and easy to perform but have great disadvantages. Generally, these simple *in vitro* 2D models are unable to mimic the complexity of the tumor tissue and therefore their cytotoxicity results are rather different from those obtained *in vivo* (Pampaloni et al., 2007).

Animal models are important to study complex interactions with surrounding cells and tissues in cancer research. However, animal models including patient-derived xenografts, are far away from genuine tumors in cancer patients. Therefore, complementary methods capable of creating a complex *in vitro* microenvironment using microfluidic technology, have emerged. Three-dimensional (3D) cell cultures were introduced in anticancer drug screening to gain more valid *in vitro* data that could more faithfully represent the *in vivo* results of drug sensitivity. 3D culturing of human cells hence mimicking the conditions of the genuine tumors can surpass both practical and ethical obstacles in using animal models. Spheroids are the simplest 3D cell culture models that mimic cell-to-cell interactions and hypoxic conditions and therefore provide a more realistic drug response than conventional 2D cultures (Costa et al., 2016). However, spheroids still have some limitations primarily including the lack of extracellular matrix (ECM), with its complex physical and chemical characteristics (Valente et al., 2017). For that reason, various scaffold-based 3D cell cultures were developed in recent years. They represent biocompatible 3D networks designed to provide structural support to the cells, with physicochemical characteristics that resemble ECM present in genuine tumor tissue (Hoarau-Véchot et al., 2018). Although these are more advanced 3D cultures, they are characterized by static conditions and lack fluid interstitial flow (i.e. blood) that is normally present in any tissue which significantly affects various phenotypes including response to cytotoxic drugs.

Other tools like next-generation sequencing (NGS) which has become irreplaceable in discovering mutations, gene expression and cancer biomarkers, offers a broad spectrum of possibilities for studying MDR (Chandana et al., 2019; Cho et al., 2019; Kyrochristos et al., 2019; Mi Li et al., 2015; Ming-hui Li et al., 2015). In the last years, high throughput NGS technology

revealed important findings regarding the genomic, epigenetic and transcriptomic diversity of cancers that otherwise would not be possible to acquire by standard histopathological analysis (Jiang et al., 2014; Teixeira et al., 2019; Turajlic et al., 2019). This is of paramount importance and clinical relevance especially for cancers displaying high levels of drug resistance (Røe et al., 2012).

Some less common techniques such as atomic force microscopy (AFM) and single live-cell imaging can facilitate the identification of MDR phenotypes in cancer patients' specimens. AFM is a very versatile tool for biological research. Its multispectral capacity to monitor the topographical, mechanical adhesive and oscillatory patterns of living cells makes the instrument highly promising for cancer research and more specifically for drug development (Prusty et al., 2018). On the other hand, the analysis performed by tracking single cells in a heterogeneous cancer cell population can determine the fate of each cancer cell while studying different characteristics including proliferative capacity, motility, shape, size, signaling patterns, and intercellular communication.

In this review we discuss the potential of i) NGS application in identification of cancer MDR as well as research covering DNA-based sequencing, RNA-based sequencing and clinical applications of NGS; ii) AFM application in cancer research and anticancer drug screening; iii) Single live-cell imaging applicability for the identification of drug-resistant and aggressive clones of malignant cells; iv) The use of microfluidics-based 3D cell cultures to evaluate cancer characteristics and complex interactions with its surrounding including blood flow as well as, v) Anticancer drug efficacy and mechanisms of resistance emerging under selective conditions that mimic the genuine tumor.

2. Next-generation sequencing (NGS) in cancer MDR research

The rapid development of sequencing technologies for the human genome and transcriptome analysis has led to an enormous increase in our knowledge regarding the roles of genetic variability in various disorders including cancer. NGS technologies are useful in a broad spectrum of applications in biomedical research. These include a variant discovery by whole-genome sequencing (WGS), whole-exome sequencing (WES) (Hitomi and Tokunaga, 2017) or targeted sequencing of genomic regions or gene panels of interest (Hlavac et al., 2018; Soukupova

et al., 2018; Wakai et al., 2019). RNA sequencing (RNA-seq) offers sequencing of the entire transcriptome and chromatin immunoprecipitation with sequencing (ChIP-seq) enables investigation of the epigenetic architecture of the genome (Rabbani et al., 2016). Other NGS applications include *de novo* assemblies of bacterial and eukaryotic genomes, species classification and/or gene discovery through metagenomics and DNA-Protein interaction analyses (Metzker, 2010; Precone et al., 2015).

Regarding MDR, NGS technologies allow us to identify DNA- and RNA-based molecular profiles associated with tumor development, progression and therapy outcome and thus recognize genetic determinants of MDR phenomenon in solid tumors (Chandana et al., 2019; Gov et al., 2017; Kyrochristos et al., 2019; Verma and Sharma, 2018) and leukemia (Albitar et al., 2017; Bereza et al., 2017; Szankasi et al., 2016). On the basis of ultra-deep sequencing, it is also possible to understand and combat the antibiotic resistome (Crofts et al., 2017; Hadjadj et al., 2019) and characterize in detail, drug resistance-associated mutations in pathogens and viruses causing serious diseases (Alidjinou et al., 2017; Gautam et al., 2019; Parker and Chen, 2017; Ramanathan et al., 2017; Soares et al., 2019; Thomson et al., 2016) as summarized in Fig.1.

2.1. DNA sequencing

DNA sequencing opens new avenues in the study of tumor aggressiveness, allows comparative analyses between different population characteristic mutation rates (Saidijam et al., 2015), evaluation of DNA damage response and repair or telomere modification attributed to chemo- and radio-resistance (Nahar et al., 2018; Røe et al., 2012). The development of chemoresistance can arise due to either single or multiple drug applications. Sequencing or copy number analysis showed that the administration of multi-agent regimens leads to the development of MDR, which appears to be pathway-specific and not dependent on the combinations of mutations (Hammond et al., 2016; Pritchard et al., 2012). In addition, it is well established that drug resistance can be induced also after targeted therapies, and therefore the necessity for a change in clinical oncology treatment strategies is emerging (Masoud and Pagès, 2017; Pritchard et al., 2012). Chemoresistance can be attributed to cancer cells with polyploidy, stemness phenotype or pro-inflammatory secretory phenotype as revealed by analysis of cDNA and mitochondrial DNA (mtDNA) (Ma et al., 2015; Rohnalter et al., 2015).

DNA repair is an important mechanism in the development of drug resistance along with chromosomal aberrations, gene amplification, and genomic deletions. Moreover, a direct association between DNA methylation and MDR has been established (Chen et al., 2015; Kadioglu et al., 2016). DNA methylation status is gaining increased importance in the diagnosis of cancers since DNA methylation status can be examined in serum, urine or sputum of patients with neoplastic or pre-neoplastic lesions (Worm and Guldberg, 2002).

Complete genome sequencing helps in the identification of genes that contribute to MDR and consequently facilitates the decision as to which therapeutic regimen the patient will benefit the most (Hearn et al., 2018; Schröder et al., 2012). Thus, identification of co-mutations such as BRAF and NRAS supports the clinical practice which uses combinational therapy to target different pathways (Raaijmakers et al., 2016). Both DNA and RNA assays remain the basic methods through which the recurrent mutations are identified in most cancer types. However, combinations of epigenomic or genomic markers combined with proteomic biomarkers increase the therapeutic success rate, rather than utilizing just a single approach (Sinha et al., 2019). An example of multi-omics approach, is the combination of proteomics, transcriptomics and DNA interactome analyses which revealed a valuable information about mutant p53 proteins that have been discovered in more than 50% of human malignancies (Walerych et al., 2016), while the role of the mutated TP53 gene in drug resistance is still speculative (Alzoubi et al., 2016).

Importantly, some mutations can have a detection rate of up to 73%, when the tests are combined from different sources: circulating tumor DNA (ctDNA), biopsy and circulating tumor cells (CTC). Although differences between CTC and ctDNA genotyping and discordant genotypes between tumor biopsy and blood-based analyses were recognized, these disagreements are due to the methodological differences in isolation (Schechter et al., 2015; Sundaresan et al., 2016). Thus, it is known that the DNA isolation for a liquid biopsy varies due to tumor-specific mutations in DNA isolated from other cells present in the urine or the fluid itself (Mikhaylenko et al., 2018).

2.2. RNA sequencing

RNA sequencing (RNA-Seq) technologies have been developed to analyze whole transcriptome profiles including gene expression profiles of protein-coding mRNA as well as non-

coding RNAs such as microRNA (miRNA), long non-coding RNA (lncRNA) and circular RNA (circRNA) profiles (Costa et al., 2013; Ng et al., 2018; Nigita et al., 2019; Z. Wang et al., 2009). This technology has advanced predominantly in the last two years and it gradually replaces whole transcriptome arrays.

In MDR research, RNA sequencing facilitates the identification of transcriptional-based markers of acquired resistance, cancer progression, and treatment response (Ming-hui Li et al., 2015; J. Wang et al., 2019; Xi et al., 2017). Besides, RNA sequencing can be used to study associations between genomic and transcriptomic alterations during tumor development, drug response and clinical outcome of patients. These changes can be associated with the development of drug resistance and differences in therapy outcomes (Cho et al., 2019). Whole transcriptome RNA sequencing can reveal the role of transcript deregulation and alternative splicing in malignant transformation and chemoresistance (De Laere et al., 2017; Goff et al., 2013) as described in Table 1. RNAs devoid of coding potential referred to as non-coding RNAs (ncRNAs) have a great impact on molecular mechanisms, ranging from developmental processes to various diseases including cancer (Beermann et al., 2016; Tomar et al., 2019). ncRNAs are divided into two subclasses according to a relatively broad size threshold –miRNAs and lncRNA.

Small non-coding RNAs called microRNAs (miRNAs) are indeed approximately 21-23 bp long RNAs, which are involved in tumor development, progression and drug resistance through regulation of gene expression. Particular miRNAs such as miR-199a-5p and miR-222 were found to be associated with MDR of human colorectal cancer (Kong et al., 2012; Xu et al., 2012), while miR-27b modulates chemosensitivity of gastric cancer cells (Fang et al., 2016). The majority of miRNA profile studies were performed using microarrays. The recent development of RNA sequencing revealed new miRNAs (miR-7-5p, miR-22-3p, miR-483-3p) the gene targets of which were correlated with drug resistance in human cancers (Jia et al., 2019; Liang et al., 2019; Xiao et al., 2018).

Long non-coding RNAs (lncRNAs) can be associated with the development of chemoresistance of multiple types of cancer (Fang et al., 2016; Liang et al., 2018; Wang et al., 2017; Wu and Wang, 2018). LncRNA transcriptome profiling has shown that lncRNAs are also involved in leukemia development, thus enabling the identification of different subtypes of human leukemia (Lammens et al., 2017). Taken together, through RNA sequencing, it is possible to detect and identify miRNAs, lncRNAs and their molecular targets as novel potential therapeutic

strategies to enhance drug response in resistant tumor cells as summarized in Table 1. Noncoding RNAs also play a critical role in the regulation of cellular DNA damage response (DDR) machinery, important for the prevention of tumorigenesis. ncRNAs can directly regulate processes involved in DDR by altering their target genes and DDR components. RNA sequencing technologies allowed the detection of interactions of ncRNAs with DDR components and render them as promising targets to overcome chemoresistance of tumor cells to conventional cytotoxic chemotherapeutics (Arjumand et al., 2018).

Circular RNAs (circRNAs) are a class of noncoding RNAs widely expressed in eukaryotic cells and characterized by their circular molecular configuration. CircRNAs were demonstrated to be potential regulators of miRNAs, transcription, and various protein functions. They can also encode for proteins, as previously reviewed (Hsiao et al., 2017; Yu and Kuo, 2019). The development of next-generation RNA sequencing technologies has allowed scientists to analyze the expression and composition of circRNA and elucidate their roles in pathological processes. CircRNA participates in the regulation of neuronal and cardiovascular diseases as well as in the regulation of cancer progression and drug resistance (Hsiao et al., 2017). Using RNA sequencing, circRNA expression profiles and their changes were identified in cancers including breast, lung, ovarian, endometrial, gastric, pancreatic, colorectal and hepatocellular cancer. Many associations of circRNA expression and circRNA-miRNA networks associated with drug resistance and cancer therapy outcomes, were found (Table 1). In addition, significant deregulation of circRNAs is correlated with drug resistance and therapy outcome of cancer patients. Bioinformatic analyses of circRNA expression identified mainly interactions with miRNA sequences (Chen et al., 2019; Huang et al., 2019). Identification of circRNAs using RNA-seq needs specific bioinformatic tools such as CIRexplorer (Galasso et al., 2016) or miARma-Seq (López-Jiménez et al., 2018) that can detect various associations and junctions of circRNAs with the transcriptome, miRNAs and other noncoding RNAs have been reported. In this respect, Kun-Peng et al., constructed circRNA-miRNA-mRNA pathways related to circRNA hsa_circ_0004674 by TargetScan and miRanda tools (Kun-Peng et al., 2018). Optimal use of RNA-seq data is currently hindered by the short-read nature of the dominant sequencing technologies, which prevents completely reliable reconstruction and quantification of full-length transcripts. Thus, it is necessary to improve the detection of long reads during RNA-seq. This is precisely why direct, amplification-free sequencing of full-length RNA molecules is being introduced with the advent of nanopore

sequencing, which raises the possibility of yet another revolution in transcriptomics in the near future (Marinov, 2017).

2.3. Targeted therapy and NGS

Tumor progression can be followed by relapse (Puig et al., 2008), due to a variety of genomic alterations, which render the cells chemoresistant, resulting in relapse. These studies suggest that a multistep pathway including DNA endoreduplication, polyploidy, followed by depolyploidization and generation of clonogenic escape cells can account for tumor relapse after initial efficacious chemotherapy. Therefore, the adaptation of new investigation strategies (Kumar et al., 2018; Siddiqui et al., 2018) such as testing patients' plasma for epidermal growth factor receptor (EGFR) mutations is ongoing. Though, decision making in treatment strategies is supported nowadays by analysis of tumor DNA extracted from the plasma of cancer patients. However, a multitude of malignant pathologies still lacks biomarkers and those currently used reveal minimal benefits in classifying patients prior to chemotherapeutic treatment (Conteduca et al., 2017).

With emerging personalized targeted treatment strategies that take into consideration differences in tumor biology of different patients (Hu et al., 2015), novel clinical approaches such as plasmid DNA vaccination, improved tyrosine kinase inhibitors (TKIs)-based therapeutics (Leonetti et al., 2019; Sundaresan et al., 2016), as well as application of new chemosensitizers (Worm and Guldberg, 2002; Wu et al., 2014), the contribution of NGS became more valuable in both identification of cancer biomarkers and in selecting the most suitable anticancer treatment modality.

Although comparative analysis showed that droplet digital PCR (ddPCR) is more sensitive and also inexpensive, the advantage of NGS is in its capacity to monitor multiple gene mutations at the same time (Yang et al., 2016). Besides, some mutations can be determined using NGS from liquid biopsies (Mikhaylenko et al., 2018). Thus, NGS significantly contributes to the selection and enhancement of current anticancer treatment regimens.

3. Atomic Force Microscopy (AFM) in cancer MDR research

3.1. Atomic Force Microscopy in biomedical research

A novel tool appeared recently among the instruments used in cancer research: the atomic force microscope (AFM). This device was invented in 1986 by the Nobel laureate Gerd Binnig to image, at high resolution, electrically insulating surfaces (Binnig et al., 1986). Since the instrument can operate in liquids, it quickly became popular among biologists. Soon after, it appeared that not only does it permit to acquire high-resolution images of biological samples in nearly physiological conditions, but it can also explore their mechanical properties (Kasas and Dietler, 2008), detect the presence of specific molecules on its surface or monitor the sample's metabolic rate. The device records all these data simultaneously and relatively simply, which qualifies AFM as promising research and diagnostic tool.

The AFM essentially consists of a sharp tip that scans the surface of the sample. The tip is fixed at the end of a soft cantilever that deflects accordingly to the topography of the scanned surface. The angular deflection of a laser beam that illuminates the cantilever end, permits to determine the vertical deflection of the lever very precisely, with a resolution that reaches 0.1 Å. Depending on the instrument's manufacturer, the tip or the sample is attached to a piezo-electric scanner and is moved along the 3 axes under the control of electric signals sent by a computer. A computer generates a 3D image of the sample's surface by displaying the cantilever deflection at each spot of the scanned sample. Fig. 2 displays the different imaging/data gathering working modes of the AFM that are of potential interest to cancer research and biomedical research.

The main motivation for AFM development was the high-resolution imaging of non-conductive samples. The very first imaging mode implemented on these instruments is the so-called contact mode since the tip is constantly in contact with the sample (Fig. 2A). However, it appeared very quickly that this mode is not appropriate to image weakly attached samples, such as individual proteins or DNA molecules. The lateral forces applied by the tip displace the sample and make it invisible to the AFM. To overcome this drawback, Zhong et al., developed the so-called tapping mode (Fig. 2B) (Zhong et al., 1993). In this mode, the tip oscillates in the vertical axis and touches periodically the sample. In this imaging mode, the tip only applies a vertical force onto the sample, preventing its lateral displacement; it is therefore well adapted to image loosely

attached molecules. The third imaging mode is the so-called non-contact mode (Zhong et al., 1993) in which the tip is set to oscillate above the sample without touching it (Fig. 2C). The change in the cantilever oscillation frequency that occurs when the tip approaches the sample, serves to calculate the tip-sample distance. Therefore, this imaging mode virtually does not interact with the sample. However, its implementation is complex and is for the moment, restricted to a few laboratories in the world.

The AFM measures mechanical properties by indenting its tip into the sample and monitoring the cantilever deflection during the process (Fig. 2D). The obtained curve, referred to as the indentation curve, displays the force that is needed to indent the tip to a given depth on the sample. Fitting the indentation curve with the appropriate model permits us to calculate the stiffness of the sample, assuming the shape of the AFM tip and some other parameters are well defined. A comprehensive and exhaustive study of this type of measurement can be found in Cappella and Dietler (Cappella and Dietler, 1999).

The detection of the presence of specific molecules expressed on the surface of the sample is achieved by attaching on the AFM tip a molecule that binds to the target (Fig.2E). By bringing the tip in contact with the sample, the two molecular species attach. During the retraction of the tip, the newly formed link deflects the cantilever downwards unless the cantilever restoring force overcomes the binding force between the two molecules. At this moment, the newly formed bond breaks and the cantilever returns to its rest position. The deflection of the cantilever before the bond breaks, multiplied by the lever spring constant, gives the interaction force between the two molecular species.

A variant of the classical tapping mode is the so-called Peak force-tapping mode. In this mode, the tip is set to oscillate at a low frequency and low amplitude to minimize the force applied onto the sample to tens of piconewtons. One single Peak force image contains information at a nanometric scale about the topography of the adhesion and the mechanical properties (elastic modulus, deformation, and dissipation) of the sample.

The last operating mode of the AFM consists of depositing the sample directly onto the cantilever (Fig. 2F). If the sample is alive, it induces nanometric scale oscillations of the lever that immediately stop as soon the organism dies. The technique is very new and permits to assess rapidly the sensitivity of bacteria to antibiotics. However, several pilot studies suggest its

usefulness in the field of cancer research and especially in the detection of anticancer drug resistance.

3.2. Imaging of cancer cells

AFM imaging mode can be very useful in several fields of cancer research. The instrument can portray single molecules or molecular complexes in near-physiological conditions. Importantly, it also monitors the structural changes induced by different drugs in real-time. As an illustration, Alonso et al., monitored the topological modifications of DNA upon exposure to doxorubicin (Alonso-Sarduy et al., 2011). At somewhat lower magnification, *ex vivo* studies performed on lipid bilayers demonstrated the mechanism of action of anticancer drugs on cancer cell membrane rafts (Corsetto et al., 2012). This capability of the AFM is also applicable at a larger scale to single cells. It permits us to study the 3D topography of cellular membranes and partially the organization of uppermost components of the cytoskeleton. Numerous similar studies explored drug effects on the cellular membrane of the whole carcinoma, colorectal, lymphoma and breast cancer cells (Huang et al., 2012; M. Li et al., 2016; Venkatesan et al., 2010; J. Wang et al., 2009). Quantifying morphological changes affecting 3D surfaces such as cellular membranes as seen by AFM can be very challenging. Simple roughness measurements are not sensitive enough to highlight subtle changes. Analyzing the fractal dimension of the cellular surface could be a valuable alternative to distinguish subtle differences occurring after drug treatment (M. Starodubtseva et al., 2017; M. N. Starodubtseva et al., 2017; Starodubtseva et al., 2019). All these data strongly suggest that following online cell surface changes upon drug action can help in distinguishing sensitive from resistant cancer cells.

3.3. Studying mechanical properties of cancer cells

Numerous links were made in the last decade between alterations of the mechanical properties of cells and pathological states. Among the studied diseases, cancer is certainly the one that focused most on the attention of AFM users. The majority of studies demonstrated that cancer cells are softer than normal cells whereas some, such as hepatocellular cancer cells, cervical carcinoma cells, myeloid and lymphoid leukemia cells were reported stiffer than their normal

counterparts (Ding et al., 2015; Lekka, 2016; Rosenbluth et al., 2006; Zhang et al., 2002). The reasons for these modifications are still unknown and subjected to debate (Rianna et al., 2018). During invasion and metastasis formation, cancer cells need to rearrange their cytoskeleton to exert enough force to break down intercellular contacts, start to move and follow their path through the extracellular matrix. These rearrangements inevitably modify the mechanical properties of the cells, since the actin cytoskeleton was demonstrated to play a major role in the determination of the cellular mechanical properties (Kasas et al., 2005). Modifications occurring in cancer not only affect cells but extend also to the extracellular matrix, which changes its stiffness and adhesive properties. The extracellular matrix not only sustains and connects cells but also influences numerous cellular functions (Ulrich et al., 2009). It is also documented to modulate cellular mechanical properties (Solon et al., 2007). By considering the mechanical properties alterations that are occurring in both, cells and extracellular matrix, it becomes possible to distinguish by AFM cancerous tissue from the normal one (Plodinec et al., 2012). The sensitivity of cancer cells to drugs can also be explored by monitoring cellular mechanical properties. Sharma et al. noticed a significant difference in the mechanical properties of cisplatin sensitive and resistant ovarian cancer cells (Sharma et al., 2012). The authors attributed the difference to changes in the actin cytoskeleton. Further studies led by Seo et al., confirmed that the acquisition of cisplatin resistance by ovarian cancer cells induces a reorganization of the actin cytoskeleton (Seo et al., 2015). Similar studies were carried on the prostate, and B-lymphoma cancer cells under various chemotherapeutic conditions and demonstrated that chemotherapeutic treatments induce an increase in the stiffness of the cells (Mi Li et al., 2015; Ren et al., 2015). These studies suggest that mechanical properties can be used to discriminate in a close future sensitive from resistant cell types. AFM could be included in the diagnostic instrumentation chain. A comprehensive recent review of nanomechanical fingerprinting of cancer can be found in Stylianou et al., (Stylianou et al., 2018).

3.4. Studying adhesive properties of cancer cells

Xiao et al., demonstrated in 2013 that not only the mechanical properties of cancer cells are useful to monitor cellular response to chemotherapy but also their adhesive properties (Xiao et al., 2013). The team compared non-small cell lung cancer cell lines (A549) with non-cancerous human primary small airway epithelial cells. The AFM measurements revealed an increased

stiffness and increased adherence of the non-cancerous population. Brief exposure to doxorubicin for 4 h in lung cancer cell lines highlighted an increase of adherence and stiffness of the cancer cells and an opposite modification of both parameters in the non-cancerous cells. This study indicates the potential of using cell adhesion modifications to assess drug activity in cancer cell lines.

More specific adhesive properties can also be explored by AFM by tracking single miRNA molecules. miRNA deregulation is observed in cancer and several cardiovascular and neurological diseases (Gebert and MacRae, 2019; Lujambio and Lowe, 2012). It was demonstrated that the levels of certain miRNA can even predict cancer prognosis (Shell et al., 2007). Thus, miRNA quantification emerged quickly as an important topic in the field of cancer research. AFM contributes to the development of this research field by detecting the miRNA in single cells without reverse transcription or amplification. The measurement consists of fixing cells, removing their plasma membrane and scanning the remaining cell components with a DNA probe attached to the tip and that is complementary to the specific miRNA (Koo et al., 2016). This type of investigation can be very useful to assess drug activity at the single-cell level and it can be speculated that miRNA monitoring can be a useful marker in the detection of drug resistance.

3.5. Characterization of extracellular vesicles by AFM

In recent years, the capacities of the AFM were applied to explore the abundance, the morphology as well the mechanical properties of exosomes and other extracellular vesicles. These organelles have a diameter in a range between 30 and 120 nm and mediate a multitude of intercellular communications (Yamamoto et al., 2019). They exhibit several surface-bound proteins and receptors. The cargo of extracellular vesicles consists of proteins and genetic material such as miRNA, mRNA proteins and DNA molecules. They are present in several body fluids such as blood, cerebrospinal fluid, saliva, urine, and amniotic liquid (Rahbarghazi et al., 2019). These organelles play an important role in numerous infectious diseases, neurodegenerative processes, and cancer (Jan et al., 2019). Compared to the traditional investigation tools such as optical and electron microscopy, AFM presents an interesting exploration tool to characterize these organelles. It can operate in a nearly physiological environment and investigate the extracellular vesicle abundance, morphology, mechanical properties, and biomolecular content. Extracellular

vesicles monitoring during chemotherapy could be an additional marker for treatment efficiency. A comprehensive review of the potential use of AFM in the field of extracellular vesicles was recently published by Sharma et al., 2018 (Sharma et al., 2018).

3.6. Using nanomotion to discriminate cancer MDR cells

It was also demonstrated that AFM-based detection of nanomotion can monitor the life-death transitions of single cells upon exposure to cytotoxic drugs (Longo et al., 2013). The technique is essentially employed for bacteria antibiotic sensitivity tests. The test lasts a dozen of minutes and is label-free. The method can be extended to cancer research to determine the sensitivity or resistance of cancer cells to anticancer drugs. The first experiments made with cancer cells (Kasas et al., 2015) were rapidly and successfully reproduced by other independent research teams. Wu et al. monitored the action of paclitaxel on the oscillation pattern of breast cancer cell lines (MCF-7) (Wu et al., 2016). Expectedly, the amplitude of oscillations dramatically dropped after the injection of the drug in the analysis chamber. The technique is relatively simple, can apply one to dozens of cells simultaneously and importantly, is completely label-free. In terms of sensitivity, in these preliminary experiments, the authors solely monitored life-death transitions. However, the technique is sensitive enough to follow subtler metabolic rate changes such as those occurring during cytoskeletal rearrangements. It, therefore, can potentially highlight subtler drug-induced effects on the cytoskeleton hence paving the way towards novel avenues for cancer research.

4. Single live-cell tracking for identification of drug-resistant and aggressive cancer cells

Single live-cell imaging has become widely used to study cancer particularly when it can address intra-tumor and inter-tumor heterogeneity and complexity. Various cell tracking methodologies and software tools progress towards quantitative analysis of the spatio-temporal dynamics of heterogeneous cell populations present in tumors. However, direct monitoring (tracking) of many initial single cells and their descendants during several days in realistic 3D tumor environments has so far not been technically feasible. Developments in lens-free microscopy may change this situation (Berdeu et al., 2018). Label-free single-cell tracking in 3D

space is not currently feasible combined with subsequent biochemical analyses of individually tracked cells while keeping their track identity. 2D single-cell tracking can more easily supplement subsequent biochemical analyses and act as surrogate measurements for the 3D situation. Holo-tomographic microscopy (HTM) may here provide high-resolution ground-truth and facilitate tracking of cellular organelles during shorter periods inside an otherwise sparse sampling regime. HTM is label-free, non-phototoxic microscopy analyzing the fine changes of a cell's refractive indices (RIs) in 3D. Hence, by combining HTM with epifluorescence, one can show that cellular organelles including lipid droplets and mitochondria show a specific RI signature that distinguishes them with high resolution and contrast. Hence, HTM may allow following the dynamics of mitochondria, lipid droplets as well as that of endocytic structures in live cells and beyond (Sandoz et al., 2018). However, the tracking of intracellular organelles normally requires both high resolution and image sampling rates that in principle could create phototoxicity and heat. One may assume that long term rapid high-resolution sampling for thousands of cells would produce an unmanageable amount of data. This in principle may not be true if the analysis takes place in real-time ("on the fly") and only accumulated data from analyses are stored. This approach has conceptual similarity to so-called compressive sampling where sensor data are compressed while measurements take place.

The advance of applications of single-cell tracking is likely to actualize a need for the design of new cell lines to improve the selection of experimental cells that are fit for tracking and analysis. For example, some types of cells tend to clump together complicating and restricting practical long term tracking. Stably transfected cell lines can help solve this problem. These cells are transfected with fluorescently tagged proteins that are commercially available. The use of chromobody technology is another alternative to visualize the localization and dynamics of cellular targets for drug screening and trace cellular processes (Traenkle and Rothbauer, 2017).

Tracking of single live cells growing as monolayers currently gave more precise insight into cellular dynamics and cell fate decisions as compared to still images taken from fixed cells (Cooper and Bakal, 2017). It provides more possibilities to explore critical biological events in cell populations, such as proliferation, migration, differentiation, cell death or to reveal rare or emergent cell sub-populations (Gascoigne and Taylor, 2008; Gómez-Villafuertes et al., 2017; Korsnes and Korsnes, 2018; Moussy et al., 2017; Piltti et al., 2018; Shaffer et al., 2017). 2D single-cell tracking is still considered technically challenging due to many experimental settings to be

considered such as cultivation conditions of distinct cell populations, appropriate cell culture density, changes in individual cell morphology, light conditions, imaging frequency, adjustment of focusing and lack of software tools (Al-Kofahi et al., 2006; Coutu and Schroeder, 2013; Meijering et al., 2012; Schroeder, 2011). The majority of approaches for cell tracking has been based on cell segmentation or frame-to-frame linking of segmented organelles (Coutu and Schroeder, 2013; Meijering et al., 2012; Schroeder, 2011). Fluorescence-based long term tracking, although commonly used, may produce phototoxicity or unwanted artifacts (Gómez-Villafuertes et al., 2017; Milan et al., 2016). Further advancement of lens-free microscopy may open new avenues for automatic 2D-based single-cell tracking (Rempfler et al., 2018).

Single-cell tracking can complement other single-cell analyses such as DNA/RNA sequencing. However, to achieve maximum combined benefit from both technologies, it is imperative that those cells previously monitored by time-lapse are later re-identified and individually collected for the secondary analysis (Gómez-Villafuertes et al., 2017). This can be achieved by using microscopes including positional coordinates, by applying fluorescent reporters for specific cells or by using chambers with labeled grids that can identify individual cells as references (Gómez-Villafuertes et al., 2017). Automatic recognition based on relative positions between cells is also a possible approach to re-identify cells.

Single-cell tracking combined with other end-point analyses such as indirect immunofluorescence has been useful to identify proteins of interest or cell division markers. Cell tracking has so far been mainly based on semi-automatic visual guidance/control or tailored recording equipment (Sato et al., 2018, 2016). There are currently only a few published results based on systematic single-cell tracking for several days showing a complete lineage tracing using standard equipment for video recording (Korsnes and Korsnes, 2018, 2017, 2015). Several studies claim the production of automatic single-cell tracking based on computer vision algorithms (Akram, S.U. et al., 2017; Huth et al., 2010; Tinevez et al., 2017). However, their contributions lack illustrations evidencing a complete lineage tracing from their recordings (Al-Kofahi et al., 2006; Davis et al., 2007; Errington et al., 2005; Khan et al., 2007; Sato et al., 2018, 2016). The presently available tools for single-cell tracking are missing methodology to resolve ambiguities such as joint multi-target tracking and classification (Hilsenbeck et al., 2016; Hormoz et al., 2016; Rapoport et al., 2011; Stadler et al., 2018). However, the development of software tracking tools is under constant improvement (Davis et al., 2007; Errington et al., 2005; Khan et al., 2007; Koh

et al., 2017; May et al., 2018). The latest advancements appear to focus on automation based on localized image analysis and recognition. Statistics from cell lineages (pedigree trees), feature extraction and identification of inheritance of traits, can contribute to discover and to resolve tracking ambiguities and thereby contribute to a holistic approach for cell tracking.

4.1. Proxy measurements from 2D light microscopy recordings

The purpose of single-cell tracking from 2D light microscopy recordings is not to mimic how they may behave at various complex environments but rather reveal the inherent properties of cells. Experiments with 3D-models can presumably help to utilize results from 2D-models for the improved prognosis of cancer. Conceptually simple single-cell tracking from 2D light microscopy recordings can provide proxy observations of inheritance of traits via measurements of cells related via pedigree trees. It can also provide information related to the process of cell division as well for example the tendency of cellular senescence for subgroups of cells due to treatments. New information is in general available when measurements from cells can be related to lineages.

The following list indicates actual types of observables from 2D light microscopy recordings and their possible biological relevance:

- Parameters from movements. A Levy flight model may fit single-cell tracking data (Huda et al., 2018). It can reflect a change in the persistence of directions of movements caused by a change in the cytoskeleton and cell shape. Stress often tends to affect the movements and motility of cells.
- Changes in cell shape can correlate with its signaling history and state (Cooper and Bakal, 2017).
- Vacuolization and vacuole inheritance may play an important role in cell death and survival.
- Nuclear changes based on tracking and extracting features from fluorescent-labeled nuclei (Bolgioni et al., 2018; Cooper et al., 2017).
- Variation in the size of pedigree trees for a population can indicate heterogeneity and potential for long term change in the composition of cells (Korsnes and Korsnes, 2018). The non-symmetric shape of pedigree trees increases the likelihood of mitotic failures.

- Correlation of cell death modalities between sister cells indicates signaling downstream pedigree trees (Korsnes and Korsnes, 2018).
- The time from observed onset of mitosis until actual cell division.
- The time between subsequent events of mitosis.
- Mitotic cell fate (Bolgioni et al., 2018).
- Changes in the texture of cell images can indicate cytoskeleton disruption or membrane rupture.
- Parameters from cell rounding (duration, size, and shape). Improper cell rounding may indicate mitotic failure (Lancaster et al., 2013).
- Contact between cells can affect them and their descendants.
- Correlations between physical contact and movements (a tendency for cells to stick together). Cells may need time to transfer information between them.
- Correlations between movements and physical contact between cells may increase the likelihood of intercellular influence.
- Synchronization of cell division among adjacent cells. Synchronous cell division may indicate intercellular communication.
- Cell rounding and its duration during mitosis. If there is no proper cell rounding or its duration is outside the normal range, then chromosomal instability may take place (Lancaster et al., 2013).

  In general, the above observables will depend on treatments and cultivation conditions. Big data analyses can support the extraction of usable knowledge from such exhaustive and presumably weakly related data. In addition, it can be used to perform data mining utilizing extensive databases containing results from many similar experiments with various toxic exposure and cell types.

Korsnes and Korsnes demonstrated correlations between parameters derived from the movements of sister cells using the computer program KoBio Celltrack (https://korsnesbiocomputing.no) (Korsnes and Korsnes, 2018). They also showed correlations between cell death modalities for sister cells following changes in cell morphology. The program visualizes search for correlations resulting in hypothesis formulation. Fig.3 is an example of such visualization; it shows A549 non-small cell lung cancer cells exposed to the marine toxin yessotoxin (YTX) at a concentration of 500 nM. The tracks of two pairs of sister cells followed

during 0-10 h after the division of their mothers are superimposed on Fig.3A. Fig. 3 also includes corresponding pedigree trees for these sister cells (Fig.3B, C). The cells belonging to the small pedigree tend finally to die (Fig. 3C) and move significantly faster than the thriving cells belonging to the large pedigree tree (Fig.3B). This recording led to the idea that observation of movements may serve as a proxy for cellular stress and inheritance (and potentially cell death in mitosis or mitotic catastrophe for some cells).

Such observations of correlations can be used for many purposes. Drug treatment of cells can affect inheritance. This implies that changes in inheritance can be considered as a response to drug treatment. Measurements of inheritance of traits may also be used for technical quality control of tracking as well as control of cell cultivation. Inheritance of signatures related to movements also has an interest in the identification of sub-populations and their level of cellular stress.

5. Microfluidic technology to study the MDR phenotype in cancer

The microfluidic technology for cell culture applications, also known as organ-on-chip, designs and fabricates microfluidic devices made of silicones, glass or thermoplastic materials (polystyrene, cyclic olefin copolymer or similar) that are structured with cameras and channels to fit the cell scale. This scale allows the recreation of complex multicellular architectures (3D cultures surrounded by epithelial barriers and/or vascular networks, etc.) and the mechanical and chemical physiological environment in a well-controlled manner. Important hallmarks of cancer and/or its TME including hypoxia, cell invasion/metastasis, and angiogenesis which can be simulated *in vitro* using microfluidic chips are highlighted in Fig. 4. These micro-physiological systems have been used to mimic some of the most relevant characteristics of tumor progression and metastasis, while cellular changes and adaptations to different environmental conditions can be easily monitored in real-time under the microscope (Hachey and Hughes, 2018; Shang et al., 2019; Sontheimer-Phelps et al., 2019).

5.1. Studying proliferative characteristics of cancer cells

One of the fundamental characteristics of tumor progression and metastasis is sustained proliferation mediated by deregulated signaling pathways accompanied by a lack of response to

growth suppressor signals. The utility of microfluidic devices to study these processes has been described recently (Hassell et al., 2017). A decrease in EGFR and phosphorylated EGFR levels was observed in lung cancer cells subjected to mechanical stress which was used to simulate the respiration process inside a microfluidic device. To achieve this, the authors designed a microfluidic device with two chambers separated by a porous and deformable membrane inducing the mechanical stimuli over the cells in a physiological manner. Another example has been described by Lang et al., who constructed a device that confronted different cell types (breast cancer cells and stromal fibroblasts) to facilitate intercellular communication (Lang et al., 2013). However, they revealed that the modified expression of estrogen receptors in breast cancer cells was not related to the heterotypic cell-cell contact. The proliferation rates of MCF-7 cells were responsible for the observed changes in the estrogen receptor expression. Another example of how microfluidic devices could simulate complex physiological proliferation processes was recently described (Ayuso et al., 2017). Proliferation was observed only in glioblastoma cells that had migrated in response to an oxygen gradient. In this case, microfluidic devices were manufactured to be impermeable to gases, forcing all the nutrients and oxygen to reach the central chamber with cells embedded in 3D hydrogel through the perfused lateral channels. When only one lateral channel was perfused, the cells far from the perfused channels migrated towards higher oxygen concentrations. No cell proliferation was observed when both lateral channels were perfused or during the migration process. Glioblastoma cells proliferated exclusively once they reached the oxygen-rich area.

5.2. Studying changes in cellular metabolism

The TME is characterized by heterogeneous zones with different physicochemical characteristics like chemical (cytokines, nutrients or oxygen) or physical gradients (stiffness). The generation of these gradients can be achieved in multiple ways in a microfluidic device (Somaweera et al., 2016). To study the effect of nutrient and oxygen gradients, Ayuso et al., cultured glioblastoma and colorectal cancer cells in a microfluidic device that is not permeable to gases in which, due to the high cell density, zones of self-induced ischemia were created by cellular metabolism (Ayuso et al., 2016). In these metabolically heterogeneous models, cancer cell sensitivity to different chemotherapeutic drugs was assessed demonstrating variations in their

effect due to the metabolic location of cancer cells. Moreover, the deregulation of the cellular energetic mechanisms present in the TME can induce the production of reactive oxygen species (ROS). The oxidative state, modulated by the antioxidant coenzyme Q10, and its relation with the cell death and resistance mechanisms have been studied by Burić et al., in a glioblastoma model (Burić et al., 2019). A significant increase in temozolomide sensitivity upon combined treatment with coenzyme Q10 was observed in drug-resistant glioblastoma cells located in the highly oxygenated zone of the microfluidic chip when compared to the hypoxic area.

5.3. Studying tumor metastatic behavior

Several reviews and research articles have recently reported different approaches that simulate the process of tumor metastasis using microfluidics (Caballero et al., 2017; Lee et al., 2016; Portillo-Lara and Annabi, 2016; Sleeboom et al., 2018; Sontheimer-Phelps et al., 2019). Hao et al., described invasion profiles of different breast cancer cell phenotypes in newly formed bone tissue inside a microfluidic device (Hao et al., 2018). Fluorescent MDA-MB-231-BRMS1^{GFP} cells showed no significant interactions with the osteoblastic tissues formed on-chip. However, in co-culture with osteoblastic tissue, MDA-MB-231^{GFP} cells increased their invasive capacity. This study opened the door to the phenotypic characterization of circulating breast cancer cells to predict the probability of bone metastasis occurring in breast cancer patients. On the other hand, several research groups have also developed multi-organ microfluidic chips to study tropism toward other organs which is characteristic of some cancers. Along this line, Alemán et al., corroborated *in vitro* the preference of colorectal cancer organoids to metastasize to liver and lung tissues (Aleman and Skardal, 2019), while Xu et al., undertook similar studies by demonstrating the tropism of lung cancer cells to nervous, bone, and liver tissues (Xu et al., 2016). Another approach in studying metastasis - vascular interaction with the tumor cells was explored (Boussommier-Calleja et al., 2019; Chen et al., 2017). Thus, Boussommier-Calleja et al., described the role of monocytes in the extravasation of tumor cells in 3D vasculature models inside microfluidic devices (Boussommier-Calleja et al., 2019). Reduction in trans-endothelial migration mediated by soluble factors released by monocytes was observed. However, macrophages derived from circulating monocytes showed no significant effect on extravasation processes. Moreover, the formation of new blood vessels has been also simulated in microfluidic devices (Haase and

Kamm, 2017). Several microfluidic designs have been employed to mimic the angiogenic process. Lee et al. described that the presence of U87 glioblastoma cells was able to markedly increase the sprout numbers and coverage areas of the microvessels compared to cancer cell-free controls (Lee et al., 2014). Agarwal et al., embedded cancer cells in alginate capsules to recapitulate the 3D structures (Agarwal et al., 2017). In a posterior step, those capsules were assembled with other cells from the TME (endothelial and stromal cells) to resemble a macroscale 3D vascularized tumor. Their results showed a very significant increase (between 4.7- to 140-fold) in drug resistance to doxorubicin when compared to the 2D culturing method. Other research groups have focused their research to test the impact of antiangiogenic drugs using microfluidic devices, providing new applications of this technology to combat cancer, particularly against chemoresistant tumors (Kim et al., 2015).

5.4. Studying interactions with the immune system

Immune system cells are also present in the TME where their role has been modulated or compromised by the tumor cells (Pardoll, 2012). Therefore, modulation of the immune response is considered a promising therapeutic strategy and recently immune-tumor interaction was evaluated in microfluidic chips (Aref et al., 2018; Moore et al., 2018). Namely, small specimens of patient biopsies were placed in the interior of microfluidic chips to assess the efficacy of immune checkpoint inhibitors. In addition, recently approved personalized therapy with genetically modified CAR-T cells was also tested in a biomimetic environment on-chip by using co-culturing of liver cancer cells with CAR-T cells (Pavesi et al., 2017). Co-culture was performed within a microfluidic device capable of controlling the environmental conditions such as the oxygen levels and the presence of inflammatory cytokines.

It is well established that inflammation affects several hallmarks of cancer and its role in carcinogenesis and cancer progression has been well established (Coussens and Werb, 2002; Kon and Benhar, 2019; Taniguchi and Karin, 2018; Todoric and Karin, 2019). Being a complex process mediated by several cell types of the TME, inflammation is not possible to study in-depth with classical culturing methods. Therefore, several approaches using different microfluidic devices to mimic the immune-cancer interactions were developed (Boussommier-Calleja et al., 2016). A

promising design to study damage-associated molecular patterns after post-ischemic inflammation has been reported (Ayuso et al., 2016).

6. Microfluidic-based 3D cell cultures for drug screening

The development of microfluidic technology and its application in designing microfluidic-based 3D cell cultures have made breakthroughs in overcoming drawbacks in evaluating anti-cancer drug sensitivity/resistance *in vitro*.

6.1. Drug resistance in microfluidic-based 3D cultures

3D cultures are generally more resistant to chemotherapeutics than monolayer cultures (Jo et al., 2018); this particularly refers to microfluidic-based cultures. In recent years, several research groups have reported this phenomenon. Ozcelikkale et al., 2017 used their TME-on-chip (T-MOC), to mimic drug transport in tumor tissue (Ozcelikkale et al., 2017). They observed that two breast carcinoma cell lines with different molecular profiles (MCF-7 and MDA-MB-231) were both more chemoresistant to doxorubicin (free or nanoparticle-loaded) in T-MOC than in 2D cultures. Moreover, triple-negative breast cancer cells, MDA-MB-231, displayed increased resistance compared to MCF-7 in the T-MOC platform. The authors revealed that the absence of eIF3a was one of the major mechanisms of drug resistance in both cell lines grown in the microfluidic chip, while overexpression of the hyaluronic acid receptor CD44 in the MDA-MB-231 cell line appeared responsible for its increased chemoresistance in T-MOC. In another microfluidic device, a butterfly-shaped microchip developed by Yildiz-Ozturk and colleagues, MCF-7 and MDA-MB-231 cells were also more resistant to doxorubicin and the antioxidant carnosic acid, than in a 2D culture system (Yildiz-Ozturk et al., 2017).

Uhl et al. showed that human colorectal cancer cells HCT116 were more resistant to conventional chemotherapeutics doxorubicin, paclitaxel, and capecitabine when grown as tumor spheroids within the bi-layer microfluidic system (Uhl and Liu, 2019). Another research group compared the responsiveness of the same cells to 5-fluorouracil, oxaliplatin, vincristine, and sorafenib in their “tumor-on-a-chip” platform and 2D culture, showing increased resistance to all tested drugs in a microfluidic device (Sobrino et al., 2016).

Another study reported that both sensitive and MDR breast carcinoma cell lines, MCF-7S and MCF-7R, were more resistant to doxorubicin in the microfluidic system compared to 2D monolayer (Sabhachandani et al., 2019). Interestingly, in 3D perfusion systems, sensitive and drug-resistant cell lines had almost the same response to doxorubicin at lower drug concentrations (1 μM and 5 μM), while drug-resistant cells become more resistant only at higher doxorubicin concentrations (10 and 20 μM)(S. Wang et al., 2019).

3D microfluidic-based cultures are also more resistant to cytotoxic drugs than static 3D cultures (Pradhan et al., 2018; Yildiz-Ozturk et al., 2017). These differences in drug response in 3D models point to the significance of the presence of media and drug flow in cell culture systems for a more clinically relevant evaluation of anticancer drug efficacy.

It is interesting to note, unlike other authors, that Jo et al., observed that glioblastoma T98G cells displayed a better response to doxorubicin in microfluidic chip compared to other static 3D cultures and even 2D cultures (Jo et al., 2018). Detailed analyses revealed that in perfusion culture, cells were almost completely removed by media flow due to their loss of cell adhesion and attachment ability upon doxorubicin treatment.

Importantly, it was shown that data obtained from microfluidic devices on drug responsiveness of both breast carcinoma and bladder cancer cell lines established from patients are similar to those collected from the corresponding *in vivo* experiments, thus confirming the validity of the use of microfluidic-based cultures in anticancer drug screening (Gheibi et al., 2017; Ozcelikkale et al., 2017).

Microfluidic technology can be also useful to separate cells based on their intrinsic characteristics, including cell size. Therefore, several microfluidic devices were developed to investigate the effect of different cell/spheroid size on drug susceptibility (Pang et al., 2016; Patra et al., 2016; Zuchowska et al., 2017). Two groups reported that both single cells and spheroids of smaller size, are more resistant to the tested drugs, vincristine, and 5-fluorouracil, respectively (Pang et al., 2016; Zuchowska et al., 2017). However, according to the study of Patra et al., the effect of spheroid size on drug response depends on the type of applied drug; smaller HepG2 tumor spheroids are more sensitive to cisplatin, while the cytotoxic effect is opposite for resveratrol and tirapazamine treatments (Patra et al., 2016).

6.2. Microenvironment effects on drug sensitivity in microfluidic-based 3D cultures

The presence of various physicochemical conditions and distinct cellular components in tumors, significantly affect the response to chemotherapy (Table 2). Microfluidic technology enabled us to introduce these tumor-related factors in cell cultures under controlled settings and to monitor their impact on drug response in a single device.

Hypoxia present in tumor tissue is one of the major TME conditions contributing to chemoresistance. Thus, its presence in 3D culture is essential to obtain more clinically relevant results on drug cytotoxicity. Microfluidic technology provides a platform for adjusting the oxygen level to perform more accurate *in vitro* drug screening. Li and colleagues designed a microfluidic chip which generates an oxygen gradient (Li et al., 2018). They confirmed its feasibility for drug screening on SF767 glioma cells and NSCLC A549 cells using two drugs differentially active under hypoxic conditions, tirapazamine and bleomycin. Another research group developed a microfluidic device to study drug resistance development under different oxygen levels (Germain et al., 2016). They showed that hypoxia-induced a rapid acquisition of drug resistance in prostate cancer cells, PC3, to staurosporine within 30 min, but this effect was reversed after switching to normoxic conditions.

The presence of media flow in microfluidic devices enables studying the effects of shear stress (normally present in tumor tissue) on drug toxicity, which is not possible to examine in other *in vitro* experimental settings. A study on a microfluidic device with two different continuous flow rates revealed that cultured ovarian cancer SKOV-3 cell spheroids developed resistance to cisplatin and paclitaxel. This shear stress-induced chemoresistance was mediated by increased expression of ATP-Binding Cassette (ABC) transporters, P-glycoprotein (ABCB1) and Breast cancer resistance protein (ABCG2), that was not observed under static conditions (Ip et al., 2016).

Apart from fluid shear stress, the mechanical effects of physiological movements could also be studied in microfluidic-based 3D cultures. Hassel et al. developed lung cancer-organ-on-a-chip that incorporates different cells, malignant (human H1975 lung adenocarcinoma cells) and non-cancerous (human primary alveolar epithelial cells and human lung microvascular endothelial cells), and which mimics physiological breathing motions (Hassell et al., 2017). The authors showed that breathing motions contributed to the resistance of EGFR mutated cells to a tyrosine kinase inhibitor, a phenomenon commonly noticed in NSCLC (Leonetti et al., 2019). The observed resistance was mediated by increased activation of EGFR and MET protein kinases.

6.3. Drug screening in microfluidic-based 3D co-cultures

The application of microfluidic platforms is particularly suitable for establishing 3D co-cultures of two or more cell types (Table 2). Microfluidic technology, with constant fluid perfusion, provides the appropriate spatial organization of different cell lines, therefore mimicking their normal physiological interactions. This is particularly important for the evaluation of drug cytotoxicity and studying the microenvironmental effects on drug sensitivity in 3D *in vitro* systems.

Several studies have shown that co-culturing cancer cells with fibroblasts in microfluidic devices induced cancer cell resistance (Jeong et al., 2016; Sabhachandani et al., 2016; Yang et al., 2018), and at the same time increased sensitivity of fibroblasts to tested drugs (Jeong et al., 2016; Sabhachandani et al., 2016). Jeong and colleagues proposed that this effect in colorectal tumor spheroids could be at least in part due to their increased fibronectin expression that coincides with the reduced drug uptake (Jeong et al., 2016).

Excluding fibroblasts, endothelial cells also contribute to the resistance of cancer cells in 3D microfluidic-based co-cultures (L. Lin et al., 2017). According to Lin et al., , resistance of cervical carcinoma cells to paclitaxel in the presence of human umbilical vein endothelial cells (HUVECs) in microfluidic chip, is associated with higher cell viability, increased expression of angiogenic proteins and active redox system (low ROS and high GSH level) (L. Lin et al., 2017).

Pericytes are another tumor-associated cell type that contributes to the chemoresistance of cancer cells in microfluidic co-culture. It was shown that spheroids of primary lung adenocarcinoma epithelial tumor cells co-cultured with primary pericytes are less responsive to cisplatin perfusion than PLETCs monoculture (Ruppen et al., 2015)

Different non-malignant cell types exert different effects on cancerous cells in 3D microfluidic co-cultures. Mi and colleagues showed that tumor-associated macrophages and monocytes contribute more to the survival of breast carcinoma cell lines in response to paclitaxel treatment in the microfluidic device when compared to epithelial cells (Mi et al., 2019).

To study paracrine interactions that confer resistance of BRAF mutated melanoma cells to the BRAF inhibitor, vemurafenib, Patel et al., developed a microfluidic device for co-culture pairs of sensitive and drug-resistant cells (Patel et al., 2015). They demonstrated that in response to

vemurafenib, drug-resistant cells exert a protective effect on sensitive cells. This effect was mediated by FGF-2/FGFR signaling and reactivation of the MAPK pathway downstream of BRAF.

Scientists and engineers have gone even a step further and developed several models of tumors/organs-on-a-chip that are comprised of complex co-cultures of malignant cells and multiple non-cancerous tumor-associated cells, such as endothelial cells, epithelial cells, immune cells and fibroblasts (Du et al., 2018; Nguyen et al., 2018; S. Wang et al., 2019). These 3D microfluidic systems authentically mimic complex tumor structures and multicellular interactions and therefore are valuable platforms for more reliable preclinical drug testing. For example, Wang and colleagues have shown that MCF-7 breast carcinoma cells are more resistant to doxorubicin in the microfluidic chip when cultured with spatially organized endothelial cells and fibroblasts than without these cells (S. Wang et al., 2019).

6.4. Single and multiple drug evaluation in microfluidic-based 3D cultures

An important aspect of every drug screening process is defining the most appropriate drug concentration/dose that will exert the expected cellular response. Microfluidic-based 3D cultures have found their application in this part of the drug discovery process as well. Several research groups have developed different microfluidic platforms for culturing cancer cells in a drug concentration gradient (Han et al., 2016; Lim and Park, 2018; K. Lin et al., 2017; Lin et al., 2019), therefore making a step forward towards rationally selecting personalized therapeutic drug dose.

These microfluidic systems, that generate spatial drug concentration gradient in long-term cultures, also allow studying the development and mechanisms underlying drug resistance. In two independent studies, the authors showed that prostate epithelial cancer PC3 cells and glioblastoma U87 cells were initially eliminated in the region of high drug concentration in microfluidic chips with the spatial drug concentration gradient. However, cancer cells soon repopulated this area due to the emergence of a drug-resistant cell sub-population and its migration to high drug concentration region (Han et al., 2016; K. Lin et al., 2017). Han et al. extracted these resistant U87 cells, which repopulated the Cancer Drug Resistance Accelerator chip upon doxorubicin treatment, and revealed that increased drug efflux activity was the main cause of their doxorubicin resistance (Han et al., 2016). They also performed exome and transcriptome sequencing and

identified several mutated genes (CHD1 and FLNA) related to doxorubicin resistance, as well as a significant number of differentially expressed genes associated with immune response, doxorubicin metabolism and NF-κB signaling. In a recent study by Lin and colleagues (Lin et al., 2019), the authors revealed that PC3 cells moved from low to high docetaxel concentration regions in which these cells were transformed into polyploid giant cancer cells, known as major mediators of therapy resistance in prostate cancer. To distinguish drug-sensitive and drug-tolerant/resistant cells, Pandya et al., developed a microfluidic device with an electrical sensing component which measures electrical conductivity changes after cell lysis in response to hydrodynamic drug flow (Pandya et al., 2017). As a cell model for system feasibility testing, they used B16-F10 mouse melanoma, 4T1 mouse breast cancer, and DU 145 human prostate cancer cells and treated them with carboplatin or paclitaxel, depending on the cell type.

Except for single anticancer drug testing, microfluidic-based 3D cultures are also suitable for the evaluation of combined drug effects, which represents a common chemotherapeutic approach in cancer treatment. Generally, microfluidic devices for multiple drug applications are designed to generate a concentration gradient of drug combinations, therefore making them useful for the optimization of combination drug treatment. For example, An et al. developed a microfluidic chip that generates 64 different concentration combinations of curcumin and tumor necrosis factor-alpha (TNFα) related apoptosis-inducing ligand (TRAIL) and tested them on PC3 cells (An et al., 2014). A different design of microfluidic chip was used by Fan et al., to examine the cytotoxic effect of 24 different concentration combinations of pitavastatin and irinotecan against the human glioma U87 cell line (Fan et al., 2016). The same group, in the recent study, improved this brain cancer chip by preventing any drug molecule diffusion between channels. The validity of the new system was tested on glioblastoma patient-derived spheroids with simultaneous exposure to temozolomide and bevacizumab (Akay et al., 2018). Most recently, Khoo and co-authors evaluated the combined effect of doxorubicin and aspirin in eliminating cancer stem cells responsible for chemoresistance (Khoo et al., 2019). They used a microfluidic array with a gradient generator component that mixes drugs and makes various drug concentration combinations.

Microfluidic-based 3D cultures could be also successfully used to evaluate the potential of different compounds to overcome MDR. As previously mentioned, Burić et al., recently reported the sensitization of resistant RC6 cells to temozolomide by its combining with coenzyme Q10 in a 3D microfluidic device with collagen hydrogel (Burić et al., 2019).

7. Future directions in cancer MDR research

Future techniques that could explain the complex interaction between resistance mechanisms and TME can help to develop efficient personalized treatment strategies. The use of phenotype-driven 3D cultures for the investigation of patient-derived biopsies could be optimal for the establishment of the most appropriate *in vivo* mimicking model. Answering the key question of which cancer patients could benefit from the specific therapeutic regimen, should be prioritized in primary tumors' treatment to avoid the development of metastatic disease. To that end, the construction of biomimetic devices with advanced characteristics may facilitate the precise manipulation of culturing conditions and sustainable maintenance of complex environmental milieu. 3D printing and microfabrication can yield scaffolds with various geometries, thus enabling spatial investigation of cancer cells co-cultured with stromal cells embedded in the specific ECM. Importantly, controlled transport of nutrients and oxygen, as well as hormones and growth factors, can provide the establishment of relevant signaling patterns in cancer cells that could not be achieved with traditional 2D culture. Besides, 3D culturing in bioreactors will secure long-term follow-up of cancer progression and treatment efficacy, thus overcoming the problem of inconsistency between the length of tumor growth in patients and tumor growth under *in vitro* culture conditions. Namely, phenotypic alterations occur during the time in a certain chronology and thus real-time monitoring should replace endpoint analyses. This will further contribute to the development of the organ-on-chip platform. One of the most successful examples is the tissue engineering of human skin for modeling human melanoma growth (Netzlaff et al., 2005). Even pharmacokinetic studies that have been exclusively performed in animal models were successfully accomplished in devices equipped with channels and chambers enabling culturing of liver, lung and fat cells in interconnected compartments with an exchange of metabolites (Esch et al., 2011). It seems rational that microfluidic devices can be adjusted for high-throughput screenings of patient samples to identify chemoresistant phenotypes and to define the most efficacious treatment regimens. This research direction will certainly favor the understanding of phenotypic changes while intracellular mechanisms could be left aside. Shifting the focus of cancer research that was for many decades directed towards studying complex signaling networks and pathways, may lead to the unravelling of key cancer research mysteries.

On the other hand, heterogeneity among cancer cells calls for their individual analysis to identify resistant and aggressive malignant clones. Heterogeneity increases the likelihood of the development of drug-resistant subpopulations which can be critical for the progression of cancer. The way to achieve the most accurate and efficacious treatment which is the administration of potent drugs to each patient, requires the characterization of cancer cell phenotypes as well as molecular profiles. The recent upgrading and continuous improvement of sophisticated sequencing tools at the single- and multiple-cell level enhance our knowledge about the molecular context of tumor behavior and interaction with its surroundings. Through new sequencing tools deciphering the molecular mechanism, underlying cancer drug resistance can help in the design of more efficient and precise chemo- and radiotherapeutic strategies. Targeted therapies were developed upon the identification of biomarkers derived from primary tumor biopsies subjected to genomic sequencing. In the future, more attention should be given to the disease in its relapsed stage and the utilization of sequencing techniques for the modification of initially used treatment regimens.

It was previously shown that cancer cells exhibit different motility patterns with the ability to shift between an epithelial to mesenchymal (i.e. amoeboid) mode to enable the invasiveness and dissemination of cancer cells (Wolf et al., 2003). Therefore, studying the motility of cancer cells using modern imaging tools can provide additional information about cancer cell behavior in association with 3D platforms. Recently, different AFM models have been created mainly to study the mechanical and dynamic properties of cancer cells (Cartagena-Rivera et al., 2015; Dufrêne et al., 2017; Guan et al., 2017). Coupled with the abovementioned complementary techniques, AFM can help to answer unresolved issues in cancer research including the interplay between drug resistance and metastatic disease. Continuous interest in the development and improvement may give AFM technology an important role in cancer research, diagnosis, and therapeutics.

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Conflict of Interest

Ignacio Ochoa Garrido is promoter and consultant for BeOnChip S.L. and EBERS Medical Technology S.L. (Zaragoza, Spain). Both cited companies have had no role in the decision to publish nor were involved in the writing of this manuscript. Mónica Suárez Korsnes declares that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest. She is the owner of the
Upstart firm Korsnes Biocomputing (KoBio; https://korsnesbiocomputing.no/) aimed to participate in research and development of methods for single-cell analysis.

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Table 1. Examples of using RNA sequencing technology in identification of MDR phenotype in human cancer

RNA	RNA sequencing technology	Effects and clinical applications	Reference
Coding RNA;			
	Targeted RNA sequencing of Androgen receptor (AR) in liquid biopsies (*n* = 34) from 30 patients with castration-resistant prostate cancer	Comprehensive AR profiling revealed associations of ***AR alterations*** with endocrine treatment outcome in patients with castration-resistant prostate cancer	De Laere et al., 2017
	RNA sequencing in patients with colorectal cancer (n=35) and multi-organ metastases	***Alterations in gene expression profiles*** during tumor metastatic development are associated with development of MDR and therapeutic outcome in colorectal cancer	Cho et al., 2019
	RNA sequencing of MCF-7 and MCF-7/MDR breast cancer cell lines	Analysis and identification of ***differentially expressed genes and associated signaling pathways involved in development of resistance*** in sensitive MCF-7 and MCF-7/MDR breast cancer cells (e.g. NOS3 and BDNF genes)	Yang et al., 2016
	RNA sequencing of CCRF-CEM and resistant CEM/ADR5000 leukemia cell lines	Except well-known MDR associated genes such as ABCB1, many ***new genes were found to be differentially regulated in MDR cells*** (ABCG2, ABCA2, Apoptosis genes, Transcription factor genes, Heat shock genes, Kinase genes, Receptor genes, Oxidative stress genes)	Kadioglu et al., 2016
	RNA sequencing of gefitinib-sensitive (PC9 and H292) and gefitinib-resistant (PC9/GR and	Gene expression profile of 48 human ABC transporters in gefitinib-resistant PC9/GR and H292/GR cells were examined using RNA-seq and compared with that of the	Zhao et al., 2018

	H292/GR) non-small cell lung cancer cell lines	parental cell lines and ***ABCC10 was found to have an important role in acquired resistance to gefitinib*** in non-small cell lung cancer	
Non-coding RNA;			
miRNA	miR-omic and RNA-sequencing of five bladder cancer cell lines	***miR-22-3p*** was selected as a target, which promotes bladder cancer resistance by targeting NET1	Xiao et al., 2018
	Small RNA sequencing and transcriptome sequencing of oxaliplatin-resistant colorectal cancer cell line HCT116/L and its parental cell line HCT116	***miR-483-3p*** was involved in the regulation of oxaliplatin resistance in human colorectal cancer cells through its functional target FAM171B	Liang et al., 2019
lncRNA	RNA sequencing data from 456 colorectal tumors compared to normal samples	Potentially oncogenic ***lncRNA SNHG15*** was found to be involved in promoting colon cancer and mediating drug resistance	Saeinasab et al., 2019
	LncRNA sequencing of the ovarian cancer cell line A2780 and the paclitaxel resistant cell line A2780/PTX	5 up-regulated and 21 down-regulated lncRNAs are considered as the multidrug-resistant lncRNAs. The multidrug resistant genes ABCB1, ABCB4, ABCC3, and ABCG2 are all co-expressed with ***lncRNA CTD-2589M5.4***.	Xu et al., 2018
	RNA-sequencing at high depth sequencing in primary follicular lymphoma samples ranging from grade 1-3A to aggressive grade	LncRNAs have been differentially expressed across the clinico-biological spectrum of follicular lymphoma with the top deregulated ***lncRNA called RP4-694A7.2*** potentially involved in cell proliferation	Roisman et al., 2019

	3B variants using unpurified (n = 16) and purified (n = 12) tumor cell suspensions from nodal samples		
circRNA	RNA sequencing of cisplatin-sensitive and cisplatin-resistant gastric cancer cells from humans	Thousands of distinct circRNAs were revealed in cisplatin-sensitive and cisplatin-resistant gastric cancer cells from humans. ***CircRNA termed circAKT3***, was significantly upregulated in cisplatin-resistant gastric cancer tissues and cells. circAKT3 modulated cisplatin sensitivity by sponging miR-198.	Huang et al., 2019
	circRNA expression profiles in normal and malignant esophageal epithelial cell lines by RNA sequencing	***813 significantly up- and 445 down-regulated circRNA candidates*** were found to be associated with pathways involved in metabolism, cell apoptosis, proliferation and migration. Interaction circRNA-miRNA network were constructed.	eCollection2017(Sun et al., 2017)

Table 2. Microfluidic devices most recently used to evaluate chemoresistance in 3D cultures depending on different environmental conditions

Microfluidic device description	Cancer cells	Environmental conditions	Drugs	Major findings	Reference
Double-layer microfluidic chip with membrane that uses chemical reaction to generate oxygen gradient	glioma SF767, lung adenocarcinoma A549	oxygen gradient	tirapazamine, bleomycin	hypoxia-induced resistance to bleomycin; normoxia caused resistance to tirapazamine	Li et al., 2018
Microfluidic system with rapid oxygen switching	prostate cancer PC3	hypoxia	staurosporine	hypoxia-induced acquisition of resistance within 30 min, with reversible effect	Germain et al., 2016
Microfluidic device with continuous, well-defined, flow rate	ovarian cancer SKOV-3 spheroids	fluid shear stress	cisplatin, paclitaxel	shear stress-induced chemoresistance mediated by increased expression of ABCG2 and P-glycoprotein	Ip et al., 2016
Microfluidic organ-on-a-chip mimicking lung airways and alveolus	non-small cell lung carcinoma H1975	mechanical stimuli	rociletinib	breathing motions contributed to chemoresistance	Hassel et al., 2017
Droplet based microfluidic platform for generation of 3D multicellular spheroids	breast carcinoma MCF-7	fibroblasts HS-5	doxorubicin	fibroblasts induced cancer cell resistance	Sabhachandani et al., 2016
4 units microfluidic chip each unit with 7 channels	colorectal cancer HT-29	fibroblasts CCD-18Co	paclitaxel	fibroblasts induced chemoresistance with increased fibroblasts sensitivity	Jeong et al., 2016,
	non-small cell lung cancer	fetal lung fibroblasts HFL1	gefitinib	fibroblasts increased chemoresistance	Yang et al., 2018

Microfluidic lung-on-a-chip with nanofiber membrane	A549				
Integrated microfluidic device with three individual components for co-culturing, protein detection and drug metabolites	cervical carcinoma CaSki	human umbilical vein endothelial cells HUVECs	paclitaxel	co-culture showed higher chemoresistance	L. Lin et al., 2017
Microfluidic device for homogeneous spheroid formation in eight replicates	primary lung adenocarcinoma cells	primary pericytes	cisplatin	higher chemoresistance in primary co-culture spheroids compared to primary monoculture spheroids	Ruppen et al., 2015
Dual-layer cell-loaded hydrogels biomimetic microfluidic system with selective permeable vascular endothelial barriers	breast adenocarcinoma MDA-MB-231	human monocytes U937, human non-tumorigenic immortalized mammary epithelial cells MCF - 10A	paclitaxel	tumor associated macrophages and monocytes more contribute to chemoresistance than epithelial cells	Mi et al., 2019
Microfluidic device with two chambers and semi-permeable gel barrier	melanoma LOX-IMVI – drug sensitive	melanoma A-375 – drug resistant	vemurafenib	resistant cells increased chemoresistance of sensitive cells by paracrine signaling	Patel et al., 2015
Microfluidic device integrating 4 cell populations with a central endothelium compartment and 2 gel compartments	HER2+ breast carcinoma BT474	human umbilical vein endothelial cells HUVECs, cancer associated fibroblasts Hs578T,	trastuzumab	CAFs contributed to resistance	Nguyen et al., 2018

		primary blood mononuclear cells			
Membrane-integrated bilayer triple-channel microfluidic chip	breast cancer MCF-7	human umbilical vein endothelial cells HUVECs, mouse embryo fibroblasts NIH-3T3	doxorubicin	cells were more resistant when cultured in the microenvironment	Wang et al., 2019

Figure Legends

Fig. 1. Application of NGS in studying MDR.

Fig. 2. Different imaging and analysis modes of the AFM. The sample is depicted in red. The arrows indicate the cantilever displacement direction. In blue: control electronics imposed motion. In red: displacement induced by the living organisms present on the cantilever (subfigure F). (**A**) Contact, (**B**) Tapping, (**C**) Non-contact, (**D**) Force spectroscopy to measure the sample's stiffness, (E) Force spectroscopy to measure molecular interaction forces, and (**F**) Nanomotion.

Fig. 3. Single live-cell tracking analysis. (**A**) Image of live A549 non-small cell lung cancer cells exposed to 500 nM yessotoxin (YTX) showing tracks of two pairs of sister cells after cell division. Black dots in the trajectory curves represent 1 h of displacement (total track 10 h). (**B**) Pedigree tree of thriving cells. (**C**) Pedigree tree of stressed cells.

Fig.4. Hallmarks of microfluidic chips application in cancer research.