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Precision Medicine in Oncology: In Vitro Drug Sensitivity and Resistance Test (DSRT) for Selection of Personalized Anticancer Therapy

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Precision or personalized medicine aims to determine an optimal therapy for each individual patient. In oncology techniques such as next generation sequencing, mRNA-sequencing, ChIP-sequencing, and mass spectrometry are used to perform a full molecular profiling for each patient. However, it is not always possible to determine a suitable treatment for an individual cancer based on molecular profiling, mostly due to the high level of tumor heterogeneity. In vitro drug sensitivity and resistance test (DSRT) can be performed on cancer cells or tissues obtained from a patient with a panel of anticancer compounds in order to experimentally define sensitivity and resistance of each individual cancer. In combination with molecular profiling, DSRT can provide a fuller picture about the nature of disease, allowing for finding more appropriate therapy for each individual patient. In this progress report, studies describing in vitro DSRTs on 2D and 3D cell models based on patient-derived cells are reviewed and challenges and future steps needed for the adaptation of these systems in clinics are discussed.

1. Cancer and its Current Treatment

Cancer is a disorder characterized by uncontrollable overgrowth of usually one particular cell type, which becomes a cancer cell, and by spreading of these cancer cells to lymph nodes and other organs of the body, what is called metastatic disease. Tissue, and in case of cancer, tumor microenvironment plays a crucial role in the process of growth and spreading of cancer cells in the body.[1]

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Cancer is an incurable disease in many cases with survival rate for >5 years ranging from 98% for testicular cancer, 90% for breast cancer, 65% for cervical cancer, 35% for lung cancer, and only 5% for pancreatic cancer,[2] making cancer the second leading cause of death worldwide after cardiovascular diseases"[3] Along with surgical removal of a tumor or radiation treatments, cancer is treated with chemo- and targeted therapy. Chemotherapeutic drugs are generally toxic for all cells of the body, with majority of drugs affecting rapidly dividing cells, therefore being more toxic for cancer cells compared to healthy ones.[4] However, for such frequently dividing cells as hematopoietic cells, cells of hair follicles, and cells lining the mouth, stomach, and intestines such drugs can be equally toxic.

Targeted therapy targets specific biomarkers, usually proteins, that cause uncontrollable growth of cancer cells.[5] Big part of targeted agents are small molecules and inhibitors, which mostly inhibit different kinases. Examples of such inhibitors include imatinib (Gleevec), an inhibitor of kinase BCR-Abl, a fused oncogene causing tumorigenesis in chronic myelogenous leukemia; vemurafenib (Zelboraf), dabrafenib (Tafinlar), and LGX818 (Braftovi) are inhibitors of mutated B-RAF, a serine/threonine-specific protein kinase causing 70% of melanoma;[6] and Gefitinib (Iressa®), which inhibits mutated epidermal growth factor receptor (EGFR) and used for treatment of breast, lung, and other cancers.[7]

Another type of targeted agents includes monoclonal antibodies, designed to attach to proteins and stimulate the immune response in the body to eliminate tumor cells expressing those proteins. Some examples of such antibodies include Trastuzumab (Herceptin), an antibody for Human Epidermal Growth Factor Receptor 2 (HER2), which is overexpressed in some breast cancers;[8] and Cetuximab (Erbitux)—a chimeric (mouse/human) monoclonal antibody against EGFR and used against colorectal, non-small cell lung and head/neck cancers.[9]

Targeted delivery of cancer therapeutics can be considered as another type of targeted therapy. In this approach, anticancer drugs are usually packed in nanocarries (NCs).[10,11] NCs aim to increase efficiency of drug delivery to tumor sites, improve stability and solubility of drugs and in some cases improve therapeutic efficacy of the drug. There are NCs that "passively" target tumor cites, meaning that they passively accumulate in tumor

tissue due to the higher permeability of blood vessels in tumor site ("enhanced permeability and retention (EPR) effect").[12] Another type of NCs functionalized with affinity ligands specific for cancer cells or tumor environment are designed to "actively" target tumors.[11]

2. Concept of Precision Medicine

Precision or personalized medicine aims to determine an optimal therapy for each individual patient. Personalized medicine emerged in early 2000s and is focusing on finding a suitable therapy for each patient by allocating a patient to a particular subgroup based on his/her characteristics, which determine to which therapy a patient will most likely respond.[13] The term "precision medicine" came first in 2009 and is used to define a treatment for each individual patient, usually using modern molecular tools as genomic profiling.[13] Precision medicine can also extend to developing an individual treatment for each patient, for example, CAR-T cell therapy.[14] Both concepts overlap in practice and often used interchangeably. In this review, we are going to use the term "precision medicine" due to its more modern and broader meaning. The concept of precision medicine is starting to be implemented for various disease types including cardiovascular disorders,[15] Alzheimer's disease,[16] diabetes,[17] and oncology.[18] Choice of precision treatment is often based on genetic profile, as well as other molecular profiling, of individual patient. Such profiling can give information about the cause, type, and stage of disease, helping to find optimal treatment for each individual patient, which is an ultimate goal of precision medicine.

3. Personalizing Cancer Treatment

In oncology techniques such as next generation sequencing (NGS), mRNA-sequencing, ChIP-sequencing, and mass spectrometry (MS) are used to determine individual profile of each patient and a tumor. Along with performing targeted sequencing to find known mutations that can be treated by existing anticancer drugs, it is possible now to perform full molecular profiling for each individual patient. Such molecular profiling includes genomic, transcriptomic, proteomic, metabolomic, and epigenomic analysis. The goal of "omic" analysis for cancer patients is to identify known variations in genome, epigenome, transcriptome, or metabolome present in the patients that can be predictive of their response to a therapy.[19–22] Pharmacogenomics is a branch of genetics, which studies the correlation between sensitivity or resistance of individual tumor to a drug and genetic background of a patient. For example, presence of particular Single Nucleotide Polymorphisms (SNP) in genome can predict response of a patient to a therapy.[23] Numerous projects have been focused on collecting databases of "omic" data, as well as software tools, and making them available for public use.[24] Examples of biggest projects are Cancer Genome Atlas (TCGA), collecting genetic mutations causing cancer; Human Proteome Project (HPP) documenting all of the proteins of healthy human body; and International Human Epigenome Consortium (IHEC) generating human epigenomes from different types of healthy and disease-related human cells. Using such databases in combination with statistical and informatics algorithms, the response of individual patients to a therapy can be predicted with certain probability based on molecular profile of a patient.[19,25–27]

Despite all these advances in individualized therapy in oncology, cancer still remains an incurable disease. There are several reasons for it. First, not all tumors carry a mutation that can be targeted with existing drugs. In a study covering sequencing of more than 3000 tumors from 26 types of cancer, it was demonstrated that only quarter of analyzed tumors contained known mutation previously associated with cancer.[28] Second, our knowledge about cancer mechanisms and databases on "omic" data are still limited. Therefore, we cannot always draw a conclusion regarding a choice of therapy based only on correlation of biomarkers. Additional challenge of full molecular profiling of individual patients is its limited applicability in clinics. These tests are usually not covered by insurance companies, leaving the costs of such tests to patients. The third and major reason for cancer being difficult to cure is its very high heterogeneity.

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Cancer includes >200 different disease entities,[29] each of which possesses high level of intratumor (occurring in one patient) and intertumor (occurring between patients) heterogeneity.[30] The total amount of mutations in one breast cancer can exceed 1000, one-tenth of which can be unique to a particular tumor and not occur in other breast cancers.[31] Moreover, a single tumor can occur from cells with different driving mutations. Kandoth et al., in their study on comparison of mutational landscape of 12 major cancer types, revealed that on average two to six driver mutations occur per individual.[32] Similarly, Stephens et al. in their study of mutational processes in 100 breast cancers found 40 different mutated genes in 73 different combinations with a number of driver mutations ranging from one to six per patient.[33] In addition, each tumor becomes more diverse during its progression. This was clearly demonstrated in a study on genomic analysis of single cancer cells derived from a single tumor[34] and in the work of Kreso et al. describing profile of ten human colorectal cancers through serial xenograft passages in mice for mutational analysis and response to chemotherapy.[35] Clonal evolution of tumors was also demonstrated in a work by Ding et al., where authors compared sequence of the primary and relapse tumors from eight acute myeloid leukemia (AML) patients, and demonstrated high rate of newly appeared mutations compared with the original clone.[36] Numerous studies revealed that high number of patients had mutations in their metastasis that were not found in the original tumor.[34,37] Frequency of appearance of new mutations depends on different individual internal (overall somatic mutational frequencies) and external (etiology) factors. For example, head and neck squamous cell tumors carry higher number of mutations when caused by tobacco, compared with tumors caused by human papilloma virus (HPV).[38] Taken together, each individual tumor is unique and carries a high level of heterogeneity. Therefore, even the same type of tumor can respond differently to the same therapy. Generally, heterogeneity and plasticity in cancer raise a risk of partial response to a therapy, when resistant cell populations stay unaffected and lead to tumor regrowth.[39] This leads to a fact that even the presence of known biomarkers does not guarantee full response to a therapy targeting those biomarkers.[26,40,41] For example, monoclonal antibodies Panitumumab (Vectibix) and Cetuximab (Erbitux) targeting EGFR, applied as a monotherapy in patients with EGFR-positive tumors, yielded only about 10% of response rate.[40] Therefore, it is important to be able to experimentally test sensitivity of a tumour to anti-cancer compounds.

In vitro drug sensitivity and resistance test (DSRT) is performed on cancer cells or tissues obtained from a patient with a panel of anticancer compounds in order to experimentally define sensitivity and resistance of each individual cancer. Utilizing DSRT in combination with information from molecular profiling can provide a fuller picture about the nature of disease allowing for finding more appropriate therapy in each individual case. Moreover, testing tumor cells with a library of anticancer compounds and their combinations can reveal sensitivity of a tumor to compounds that are usually not prescribed for this type of cancer, so-called drug repurposing.[42–44] Finally, repeated test on tumor cells obtained from a patient after the therapy, can identify resistant cells that were not eliminated with the first therapy, and find compounds that can be effective against them.[44]

Ex vivo tests on primary patient-derived tumor samples include xenograft models, tumor tissue slices, and 2D and 3D cell culture models.[45,46]

1) In xenograft models, fresh pieces of tumor are implanted into immunodeficient mice or chicken egg chorioallantoic membrane (CAM) with a goal to follow the dynamics of tumor progression during and after treatment.[47,48] Xenograft models are very important for pre- and co-clinical evaluation of anticancer treatments.[49] However, there are some drawbacks of xenograft models, which restrict their clinical applications for making decisions about the appropriate therapy. First, the success rate of establishing of a xenograft is low; second, the time of establishing varies from 2 to 12 months; third, this method is very costly and not compatible with high throughput required in precision medicine.[45,47]
2) Tumor tissue slices are thin slices of a tumor that are tested in microtiter plates with different compounds. The advantage of this model is that tumor cells are preserved in their original environment and their response to drugs can closely represent tumor response in vivo. The model is, however, only limited to patients that undergo a surgery. Moreover, this is a low throughput method limiting the number of possible compounds and combinations that can be tested.[45,50]
3) In vitro 2D and 3D cell culture models are most promising to be adopted for testing of patient-derived cells in clinics, due to their compatibility with high throughput, possibility to be performed within 2–3 days, and requirement of relatively low cell numbers. Performing such in vitro sensitivity tests as a routine in clinical practice will open a new era of precision medicine in oncology and will help to navigate the decision making toward successful therapy for each individual patient. In this paper, we review studies describing in vitro DSRTs on 2D and 3D cell models based on patient-derived cells and discuss challenges and future steps needed for the adaptation of these systems in clinics.

Despite the progress in sequencing and other "omic" analysis techniques, it is still not possible to accurately predict response of a particular patient to a therapy based on data of molecular profiling. Therefore, experimental test (DSRT) performed on cancer cells of the patient to elucidate individual sensitivity and resistance to different therapies can help defining suitable treatment for each patient, decreasing a risk of adverse effects, and development of resistance to the therapy.

4. DSRT on 2D Cell Culture Models

First DSRT of patient-derived tumor material dates back to the 1950s.[23,51,52] A lot of studies conducting in vitro tests on tumor cells from patients with leukemia and solid tumors were published in the period from 1970s to 1990s.[23,53] At that time, before molecular profiling was established, such tests were the first steps toward precision medicine in oncology. From early 2000s, with developing of sequencing technologies, genomic and transcriptomic profiling of tumors came forward. With a premise that molecular profiling and targeted therapy would solve the problem of non-responding malignancies, DSRT was less frequently

used. However, there is now more understanding that combination of both approaches can give the fuller picture about a profile of an individual disease. In this review, we will mostly focus on studies published after 2000.

DSRT on 2D cell culture models is performed on tumor cells obtained ether from blood or bone marrow of patients with blood cancer or from pieces of solid tumors, which is usually disintegrated into single cell suspension before testing. Obtained cells are plated in microtiter plates and treated with a range of concentrations of anticancer compounds for 24 to 72 h depending on cell type and read-out assay. Commonly, such assays are performed in 96-[27,54] or 384-well plates[21,43,55] using 50 000–200 000 or 5000–20 000 cells per well, respectively. Cytotoxicity of drugs is estimated either by evaluating the ability of the tumor cells to proliferate and form colonies, or by counting the number of live and metabolically active cells per well.[56] Colony-forming assay is based on evaluating the ability of cells to form colonies after 2–3 weeks on agar medium culture.[57] Number of live cells can be estimated by measuring cell metabolism using bulk solution methods such as the tetrazolium (MTT) assay,[54] CellTiter 96 AQ_{ueous} One cell proliferation assay,[27,42] the fluorometric microculture cytotoxicity assay (FMCA),[51,58] and CellTiterGlo assay.[21,43,44,59] The MTT assay is a colorimetric assay that assesses cell metabolic activity by reducing the tetrazolium dye MTT by NAD(P)H-dependent cellular oxidoreductase enzymes to form colored formazan.[60] CellTiter 96 AQ_{ueous} One assay is similar to the MTT assay.[60] FMCA is based on measuring fluorescence, which is generated by hydrolysis of fluorescein diacetate (FDA) by cell esterases.[61] CellTiter-Glo assay measures the level of ATP produced by live cells using firefly luciferase.[62] Cell viability can be also quantified by staining cells followed by microscopy.[55] Hoechst 33 342 and 4′,6-diamidino-2-phenylindole (DAPI) are often used to stain cell nuclei and estimate total cell number.[63,64] There are a number of viability dyes available including CyQuant,[55] Calcein AM, and propidium iodide that can distinguish between live and dead cells. Since majority of chemotherapeutic drugs induce apoptosis in cells, staining apoptotic cells, for example, with Annexin V,[65] which binds to phosphatidylserine externalized on outer membrane during apoptosis, or using TUNEL assay,[66] which detects damaged DNA in cells, are often used. Both of these methods are based on analysis of stained cells either by microscopy or fluorescence-activated cell sorting (FACS). In addition, using microscopy-based read-out opens possibilities for more in-depth analysis of treated cell population. For example, performing immunostaining with antibodies against specific marker of cancer cells enables distinguishing between response of cancer and healthy cells, identifying if drug is specific or generally toxic.[64]

5. DSRT of Blood Cancer

Studies on DSRT introduced in this review are summarized in Table **1**. Majority of these studies are focused on different types of blood cancer.[21,22,27,42–44,54,55,58] The reason for this is relatively large number of cells that can be obtained from bone marrow biopsy or blood samples (between 10^4 and 10^7 from bone marrow biopsy;[67] and up to 10^6–10^7 cells from blood sample). This is also reflected in the number of patients recruited in such studies, as well as number of anticancer compounds that can be tested with a sample from a single patient (Table 1). There is a number of studies performing screenings on primary leukemia cells with large panels of drugs containing from 50 to 450 compounds.[21,43,44,59] Below are some examples of studies performing sensitivity and resistance testing on cells from patients with blood cancers.

Larsson et al. tested cells obtained from blood and bone marrow of 44 patients with AML in 96-well plates against ten anticancer compounds. Toxicity of compounds was estimated by using FMCA. The results from DSRT was positively correlated with patient response to tested compounds in clinic.[58] Yamada et al. in their study used leukemic cells from 132 children with AML and found correlation between clinical non-responders and resistance of cells in vitro, as well as between subtypes of AML according to French–American–British classification and sensitivity of cells to particular drugs.[68] Maxson et al. in their study combined sequencing of leukemia cells obtained from patients with chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (CML) with drug sensitivity testing performed on murine cells transduced with particular mutations. In vitro test was performed against a small-molecule kinase inhibitor library combined with a library of small interfering RNAs. For estimation of cell sensitivity, primary-cell colonies-forming assay was used.[22] The authors sequenced samples from 27 patients and showed that 59% of patients carried activating mutations in gene of colony stimulating factor 3 (*CSF3R*) resulting in increased signaling through JAK kinases. The authors performed DSRT on primary cells obtained from one patient with ruxilitinib. As expected from mutagenesis profiling, the cells were sensitive to ruxilitinib treatment in vitro, which was confirmed by strong positive response of this patient to ruxilitinid therapy.[22] In a follow up study, Maxson et al. performed the screen on primary cells from patients with AML and chronic myelomonocytic leukemia (CMML) against a panel of kinase inhibitors using CellTiter 96 AQ_{ueous} One solution cell proliferation assay.[69] The outcome of functional screen was combined with data obtained from genomic profiling of the same specimens. A HitWalker algorithm was used to find correlations between mutational and drug-sensitivity profiles. Using this methodology authors were able to spot new correlations between mutations in gene of kinase TNK2 and sensitivity to existing multikinase inhibitor dasatinib and TNK2 inhibitors XMD8-87 and XMD16-5.[69] Tyner et al. experimentally defined sensitivity of tumor cells from 151 leukemia patients against 66 small-molecule kinase inhibitors.[27] For assessing cytotoxic effect of drugs they used CellTiter 96 AQ_{ueous} One solution cell proliferation assay. They clearly demonstrated that in vitro drug sensitivity test predicted the clinical response and even the development of drug resistance. Obtained functional data on individual drug sensitivity and known targets of tested kinase inhibitors were used to develop an algorithm, which enabled prediction of patient sensitivity to kinases based on their genomic data.[27] Frismantas et al. tested 60 anticancer compounds on 68 samples obtained from acute lymphoblastic leukemia (ALL) patients using an imaging-based cell viability readout. In this read-out cells were stained with CyQUANT dye that stains nuclear DNA. Functional in vitro testing was performed in parallel with targeted sequencing of 52 frequently mutated genes in ALL.[55] The authors found no correlation between observed in vitro drug responses and data

Table 1. A table summarizing published studies on DSRT organized by type of cancer, using 2D and 3D cell models and utilizing state-of-the-art platforms and alternative miniaturized systems.

Type of cancer	Number of cells per well	Format	Assay	Number of patients	Number of drugs	Predictability of clinical response	Reference
			2D cell models				
			Blood cancers				
Acute myeloid leukemia (AML)	50 000	96-Well plate	FMCA	44	10	Predictive (78%)	[58]
Acute myeloid leukemia (AML)	20 000	96-Well plate	MTT assay	132	13	Predictive of short-term, but not of long-term response	[68]
Chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (CML)	25 000	N/A	Primary-cell colonies-forming assay	1	1	Predictive	[22]
Leukemia	50 000	96-Well plate	CellTiter 96 AQ_{ueous} One solution cell proliferation assay	151	66	Predictive (1 patient)	[27]
Acute lymphoblastic leukemia (ALL)	30 000	384-Well plate	Imaging-based cell viability readout	68	60	Predictive	[55]
Leukemia and lymphoma of B cell, T cell, and myeloid origin	20 000	384-Well plate	ATP-based CellTiterGlo assay	246	63	Predictive	[21]
Acute myeloid leukemia (AML)	10 000	384-Well plate	ATP-based CellTiterGlo assay	18	187	Predictive	[44]
			Solid tumors				
Breast cancer	30 000	96-Well plate	FMCA	37	6	Predictive with sensitivity 89% and specificity 53%	[51]
Breast cancer	100 000	96-Well plate	MTT assay	175	10	Predictive 47%	[74]
Ovarian cancer	80 000	96-Well plate	MTT assay	32	6	Not assessed	[71]
Glioblastoma multiforme (GM)	N/A	N/A	Flow cytometry-based detection of apoptosis	40	30	Predictive 26%	[63]
Gastric cancer using	100 000	96-Well plate	MTT assay	435	7	Not assessed	[75]
Ovarian adenocarcinomas	500 000	12-Well plate	BH3 profiling	16	1	Predictive	[76]
Chronic myeloid leukemia (CML)	1 000 000	12-Well plate	BH3 profiling	24	1	Predictive	[76]
Head and Neck cancer	1000	96-Well plate	MTT assay	3	11	Predictive (1 patient)	[79]
Acute myeloid leukemia, non-Hodgkin's lymphoma and ovarian cancer	N/A	N/A	Apoptotic drug sensitivity assay	59	7	Not assessed	[81]
Acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelocytic leukemia (CML), lymphoma, multiple myeloma, breast cancer, colon cancer, non-small-cell lung cancer(NSCLC), ovarian cancer and renal cancer.	5000 (solid tumors) 12 000-40 000 (leukemia)	384-Well plate	FMCA	100	14	Not assessed	[83]
Ovarian and peritoneal cancer	2000–10 000	96-Well plate	ATP-based CellTiterGlo assay	4	240	Not assessed	[124]
			Miniaturized systems				
Multiple myeloma (MM)	7500	MicroC3 Microfluidic-based	Imaging-based cell viability readout	17	1	Predictive	[81]

(*Continued*)

Table 1. Continued.

Type of cancer	Number of cells per well	Format	Assay	Number of patients	Number of drugs	Predictability of clinical response	Reference
Breast cancer patients	1000	96well-formatted Micro-gap Plate	Imaging-based cell viability readout	3	2	Not assessed	[88]
Pancreatic cancer	100	Droplet microfluidic based	Detection of apoptosis by measuring caspase-3 activity	4	10	Not assessed	[87]
Chronic lymphocytic leukemia (CLL)	100	Droplet-Microarray	Imaging-based cell viability readout	5	9	Not assessed	Under review
Breast cancer (CTCs)	1000		Imaging-based cell viability readout	24	1	Not assessed	[114]
			3D cell models				
Colorectal cancer	250 spheroids per drug	Indi-Treat™ array	Imaging-based cell viability readout	5	4	Not assessed	[98]
Head and neck squamous cell carcinoma (HNSCC) established PiCa cell line	2000–5000	Ultra-low attachment (ULA) 96 well plate	Imaging-based cell viability readout	1	3	Not assessed	[99]
Ovarian cancer	2500	96-Well plate	Imaging-based cell viability readout	>1	22	Not assessed	[102]
Colon cancer	5000	384-Well plate	CellTiter Glo assay	4	1	Not assessed	[103]
	1000–3000	96-Well plate	CellTiter Glo assay	4		Predictive	[104]
Colorectal and gastroesophageal cancer	4500–6000	96-Well plate	WST8 viability assay	71	55	Predictive	[105]
Melanoma	20 000	96-Well plate	CellTiter Glo assay	38	3	Not assessed	[125]
			Miniaturized systems				
Non-small cell lung adenocarcinoma	5000	Microfluidic chip	Caspase-Glo®3/7 assay	2	1	Not assessed	[107]
Mesothelioma patients	N/A	Microfluidic chip	Imaging-based cell viability readout	2	4	Predictive	[108]
Lung cancer	N/A	Microfluidic chip	Imaging-based cell viability readout	8	2	Not assessed	[109]

from genetic profiling. Interestingly new sensitivities to compounds were found through functional in vitro sensitivity assay. A patient, whose cells showed unexpected sensitivity to dasatinib, demonstrated complete response to this compound.[55]

Dietrich et al. conducted a large study profiling in vitro drug sensitivity of cells obtained from 246 patients diagnosed with blood cancers against a panel of 63 drugs in vitro using cell viability ATP-based CellTiterGlo assay. Results of DSRT were combined with genome, transcriptome, and DNA methylome analysis (**Figure 1**).[21] The main goal of the study was to find genotype–phenotype associations in order to better understand the mechanism underlying drug sensitivity and resistance and to be able to use such correlations to predict suitable therapy based on genomic analysis. Their results demonstrated that patients with similar responses accurately clustered into groups of similar mechanisms of action. They were able to predict clinical outcome of the treatment based on in vitro sensitivity testing and noted consistency between results of DSRT combined with data from molecular profiling and clinical outcome.[21]

Pemovska et al. and Kulesskiy et al.[43,44,59] from the Finland Institute of Molecular Medicine (FIMM) introduced the individualized systems medicine (ISM). The main goal of ISM was to identify suitable cancer drug therapy for each patient.[44] ISM includes the following parts: i) molecular profiling and in vitro DSRT, ii) clinical implementation of therapies predicted to be effective, and iii) studying consecutive samples from the treated patients to understand the basis of resistance.[44] In the work of Pemovska et al., authors tested cells obtained from 28 patients with AML against a panel of 187 anticancer compounds. Similar to the study of Dietrich et al.,[21] the authors of this study were able to cluster patients into five groups according to their drug sensitivity and found correlations of these groups with mutational profiling. However, not all clusters defined by drug sensitivity test correlated with mutations found from genomic profiling. The authors demonstrated that DSRT was predictive of clinical responses according to European LeukemiaNet response criteria. In addition, they were able to spot development of drug resistance and sensitivity after the treatment. Repeated DSRT on cells obtained from one of the patients after the treatment showed that cancer cells gained resistance to dasatinib and rapalogs and became sensitive to certain tyrosin kinase inhibitors. Deeper investigation of a resistant sample demonstrated that resistance most likely did not occur due to a novel genetic alteration, but developed due to the preselection of existed sub clones of cancer cells. Thus, the authors were able not only successfully predict clinical outcome based on DSRT, but also identified development of

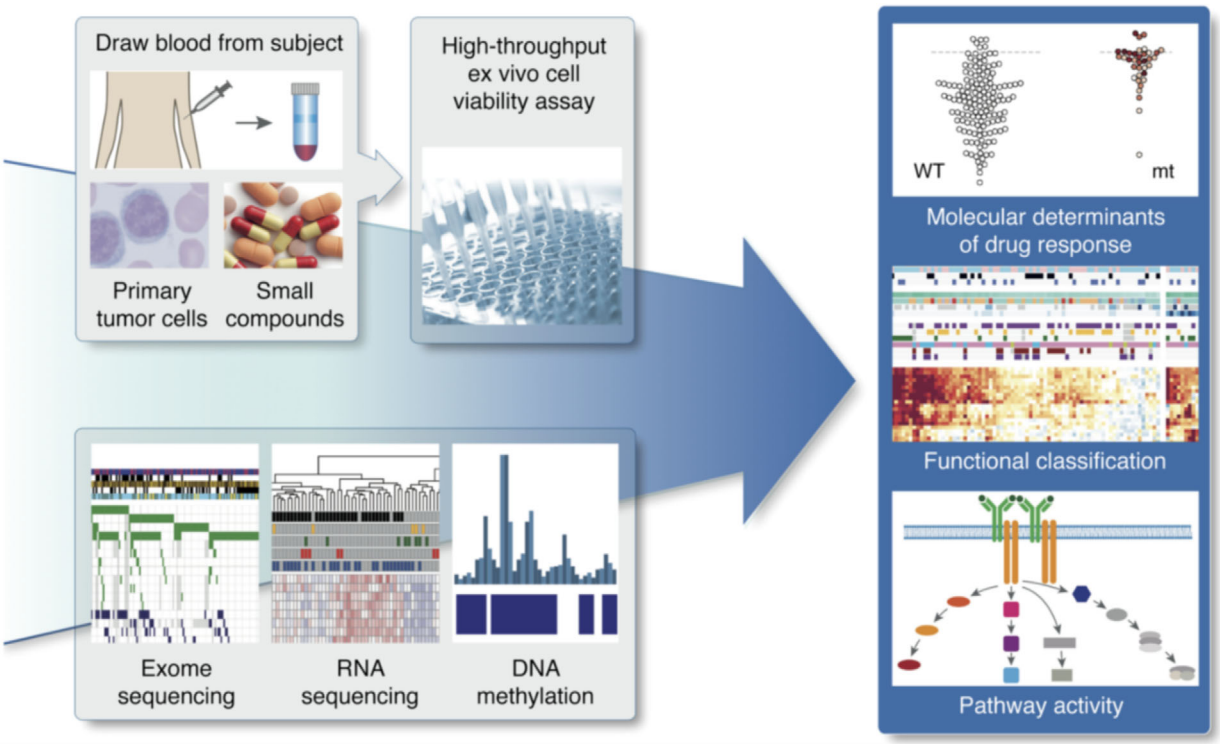

Figure 1. Scheme summarizing methods used in precision oncology on example of blood cancer. Combining functional drug response screening with omics profiling allows for systematic query of drug response phenotypes, underlying molecular predictors, and pathway dependencies of leukemia and lymphoma. Reproduced with permission.[21] Copyright 2018, American Society for Clinical Investigation.

resistance after the therapy.[44] In an article by Kulesskiy et al., the workflow of DSRT platform with 450 anticancer drugs on patient-derived cells using the acoustic nano-dispenser was described in details.[59] In the following work of Pemovska et al., authors performed DSRT on cells obtained from patients with CML and Philadelphia-chromosome-positive (Ph1) ALL patients using 252 drugs.[43] With this comprehensive screening, they defined axitinib as a selective BCR-ABL1(T315I) inhibitor and demonstrated that it is effective in patients with BCR-ABL1(T315l)-associated disease.[43]

Snijder et al. developed a concept of pharmacoscopy, which is a methodology for ex vivo drug response profiling.[64] This methodology is based on immunofluorescence, automated microscopy, and image analysis of biopsies cells introduced to anticancer drugs in vitro. The main goal of pharmacoscopy is to identify specific response of marker-positive malignant cells to anticancer drugs. According to the study, pharmacoscopy could predict the clinical response of 20 AML patients to initial therapy with accuracy of 88.1%. In addition, this methodology was used to profile 48 patients with aggressive hematological malignancies, 17 of whom received the pharmacoscopy-guided treatment resulted in partial and complete remissions. Pharmacoscopy is evaluated in ongoing clinical trial at the moment.[64]

6. DSRT of Solid Tumors

DSRT on solid tumors is more challenging compared to blood cancers due to limited number of cells available for the test and a need for harsh enzymatic disintegration of a tumor. In most studies, cells for in vitro tests were obtained after surgical removal of the tumor. This restricts applicability of sensitivity profiling only to patients undergoing surgery. Number of cells usually obtained with non-invasive needle biopsy is about 500 000 to 1 000 000 cells.[70] Considering that big part of patient material is used for other tests including pathology and genetic profiling, there is not enough cellular material left for DSRT. This is reflected in a fact that only up to 30 compounds are usually tested on each single patient sample with majority of studies evaluating less than ten compounds per patient (Table 1).[51,71–73] There are several examples of studies conducted on cells obtained from different types of solid tumors that are summarized in Table 1 and described below.

Villman et al. tested cells obtained from tumor biopsies of 37 patients with breast cancer using FMCA.[51] The result of DSRT obtained in vitro was then correlated with clinical outcome. The authors demonstrated that in vitro test predicted in vivo response with a sensitivity (correlation of in vitro test with drug sensitivity in patients) of 89% and a specificity (correlation of in vitro test with drug resistance in patients) of 53%. They also observed that low drug resistance in vitro was strongly associated with longer time of tumor progression.[51] Xu et al. investigated in vitro sensitivity of breast cancer cells from 175 samples against ten anticancer drugs using the MTT assay. The results obtained from 83 (47%) samples showed positive correlation with clinical outcome, which was assessed according to standard World Health Organization (WHO) criteria.[74] Brigulova et al. tested tumor cells from 32 patients with ovarian cancer against a panel of six anticancer

compounds using the MTT assay.[71] Authors compared in vitro sensitivity of tumor cells isolated from surgically removed primary tumors and tumor cells isolated from ascites of the same patients. The ascites are abnormal accumulations of fluid in an abdomen occurring with multiple types of cancer and containing tumor cells infiltrating from original tumor site. Obtaining cells from ascites is less invasive and painful for the patients, and protocol for cell isolation is easier to perform resulting afterward in higher cell viability in vitro comparing to viability of cells isolated from disintegrated solid tumors. Obtained results showed that, except for paclitaxel, cells from both sources demonstrated identical dose-response to the tested drugs. This result shows possibility of performing sensitivity profiling on cells from ascites instead of cells from the original tumor.[71] Iwadate et al. investigated the feasibility of performing DSRT for patients with glioblastoma multiforme (GM).[63] Cells obtained from 40 patients diagnosed with GM were tested against a panel of 30 anticancer compounds using flow cytometry-based detection of apoptosis. Authors concluded that prescribing chemotherapy for GB patients recommended on results of in vitro sensitivity testing is reasonable, although a larger study is required to draw final conclusion.[63] Noguchi et al. analyzed large number of tumor samples isolated from 435 patients with gastric cancer.[75] Cells were obtained from 485 lesions including 415 primary tumors and 70 metastatic tumors. Specimens of metastatic tumors were obtained from liver, lymph nodes, ovaries, and malignant ascites. Interestingly, authors noted that in vitro chemosensitivity of metastatic tumors was lower compared to sensitivity of the primary tumors. In addition, tumor cells obtained from stage IV cancers were on average less sensitive to drugs in vitro compared to specimens from stage I, II, and III cancers, which correlated with clinical observations. The authors compared the outcomes and predictability of DSRT and clinicopathological findings, such as tumor differentiation, macroscopic appearance, and depth of invasion. The authors found no correlation between drug sensitivity of tumors in vitro and their pathological characteristics, and concluded that it is difficult to predict clinical sensitivity of a tumor based on pathological analysis. Overall, it was concluded that in vitro sensitivity test on both primary and metastatic tumors repeated during disease progression, is essential for defining a suitable chemotherapy regiment for each individual patient.[75] Montero et al. in their study suggested and investigated an interesting approach for predicting cancer response to chemotherapy by using BH3 profiling of primary patient-derived cells pre-incubated with anticancer compounds.[76] BH3 profiling is based on measuring of mitochondrial membrane potential ($\Delta\Psi m$) by using fluorescent dyes that produce $\Delta\Psi m$ dependent shifts in fluorescence.[77] Change in mitochondrial membrane potential is caused by mitochondrial outer membrane permeabilization (MOMP) induced by incubating compound-pretreated cells with BH3 peptides. BH3 peptides interact with BCL-2 family of proteins that regulate commitment of the cell to mitochondria-dependent apoptosis pathway. In other words, sensitivity of mitochondria to BH3 peptides indicates initiation of apoptosis pathway in pretreated cells. Such test can show sensitivity of tumor cells to chemotherapy within 16 h instead of several days. The authors demonstrated that BH3 profiling predicts chemotherapy response across many cancer types and many agents. They also tested 16 primary ovarian adenocarcinomas from surgical resection and found that percentage of "priming" (or high sensitivity of mitochondria to BH3 peptides) correlated with patient response to therapy in clinic.[76] Cortese et al. developed an ex vivo chemotherapy drug response assay (ChemoID),[78] based on assessing drug response of Cancer Stem Cells, as well as bulk tumor cells in 96-well plates using MTT assay.[79] The test was performed in certified laboratory. In addition, protocols for sample collection from the patients and its preservation for international shipment were developed. In this study, specimens from three patients with head and neck cancer were analyzed.[79]

In an extended DSRT, not only sensitivity of tumor cells to anticancer compounds can be tested, but also toxicity of the same compounds to healthy cells of the body. From estimating of LC90 (lethal concentration of drug killing 90% of cell population) of compounds for both tumor and healthy cells, so-called therapeutic index is derived, which corresponds to a ratio of LC90 of normal cells to LC90 of tumor cells. Therapeutic index is usually utilized in drug development pipeline to estimate efficacy and safety of drug candidate.[80] However, there are very few studies demonstrating the importance of using therapeutic index to determine suitable drug and the window of its concentrations, when it shows efficacy but not toxicity, for each individual patient.[81–83] Bosanquet and Bell in their work tested tumor and normal cells obtained from 59 patients with different types of cancer including acute myeloid leukemia, non-Hodgkin's lymphoma, and ovarian cancer.[81] In 73% of tested tumors with purity of tumor cells being <90% after isolation, healthy cells were tested in this mixed co-culture and distinguished from tumor cells by morphology. In remaining 27% of tested specimens, which were isolated with >90% of tumor cells, healthy cells were isolated from blood of the same patient and incubated with drugs separately. In **Figure 2**, sensitivity profiles of tumor and healthy cells from one patient to caclophosphamide, fludarabine, and vincristine are shown. Caclophosphamide and fludarabine have unfavorable therapeutic index (0.3 and 1.07), because these drugs show almost identical toxicity to tumor and healthy blood cells. Vincristine, on the other hand, has favorable therapeutic index (169), because its dose-dependent effect on healthy cells is shifted two orders of magnitude toward higher concentrations of the drug leaving wide concentration range, when it is effective against tumor cells and not harmful for healthy cells. In Figure 2b, the sensitivity profiles of tested patients against a panel of anticancer compounds are plotted. The graph shows that working concentrations of anticancer compounds vary drastically between the patients. In addition, the window of concentrations where the drug is effective against tumor and still not toxic to healthy cells is very narrow or does not exist. The only way to determine this window is by performing DSRT on both malignant and normal cells. Unfortunately, most cytotoxic chemotherapy is given at or near the phase I—determined maximum tolerated dose without distinguishing between individual patient sensitivities. The authors have clearly demonstrated that knowing therapeutic index of a drug for each individual patient is extremely important not only for defining the drug that is efficient against individual tumor and not toxic to normal cells, but also for identification of suitable concentration of a drug.[81] In another work, Haglund et al. demonstrated the importance of using tumor and toxicity panels together for predicting individual drug effects.[83] Authors have tested more than 100 tumor

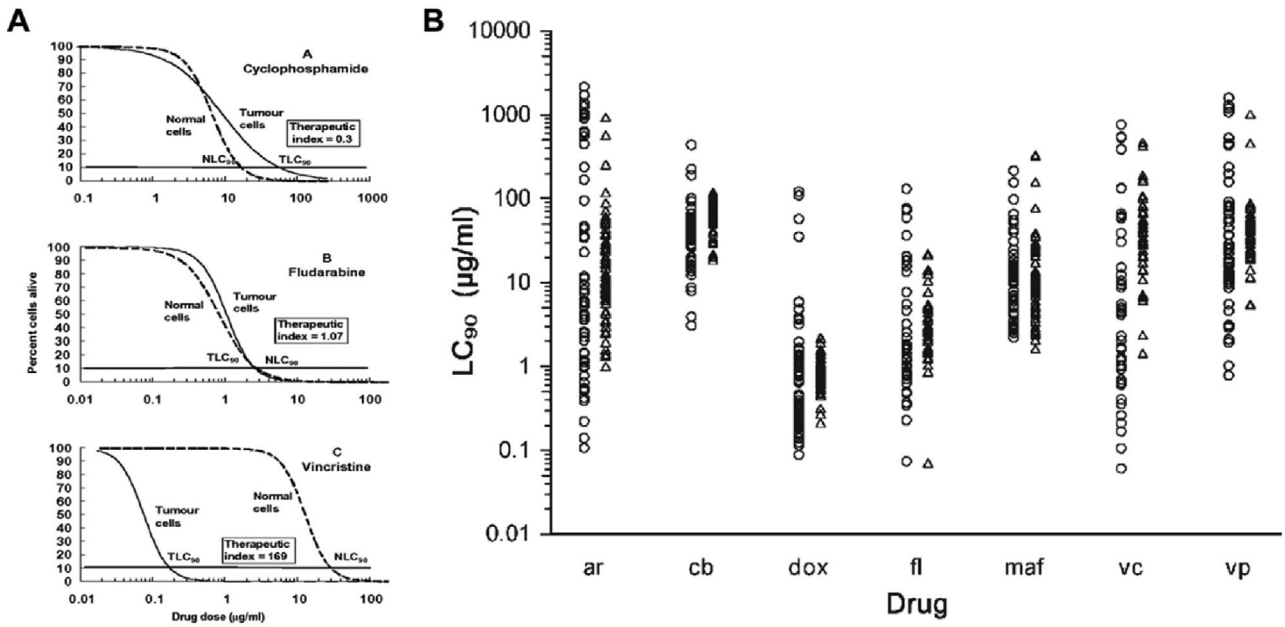

Figure 2. Comparison of dose-response of tumor and normal cells. a) Cell survival and calculation of therapeutic index in a previously treated patient with mantle cell non-Hodgkin's lymphoma. Upper panel: unfavorable therapeutic index for cyclophosphamide (mafosfamide in vitro). Middle panel: therapeutic index of ≈1 for fludarabine. Lower panel: favorable therapeutic index for vincristine. b) Scatter of LC90s for tumor and normal cells. Circles, tumor cell LC90s; triangles, normal cell LC90s; ar, cytarabine; cb, carboplatin; dox, doxorubicin; fl, fludarabine; maf, cyclophosphamide (mafosfamide in vitro); vc, vincristine; vp, etoposide. Adapted with permission.[81] Copyright 2004, Old City Publishing, Inc.

samples from patients with solid and hematological tumors along with assessment of normal tissue toxicity. They used lymphocytes (PBMC) from healthy donors to reflect hematological toxicity and human epithelial and renal cell lines to reflect epithelial and renal toxicity, respectively. They were able to detect drugs with wide and narrow therapeutic index, which correlated with known side effects of these drugs in clinics.[83]

There is a substantial number of publications about DSRT from the 1950s to our days, which shows importance of this method for prediction of individual drug sensitivities and resistance. Standard protocol for DSRT is performed in microtiter plates requiring large number of patient cells. That is why most of the publications is focused on blood cancer, where high number of patient cells can be obtained. Nevertheless, there are a number of publications on DSRT performed on solid tumor cells as well, in which mostly small number of drugs can be tested. In order to be able to test all tumor types with large drug libraries, it is necessary to miniaturize DSRT using platforms alternative to microtiter plates.

7. Miniaturized Systems for DSRT on 2D Cell Culture Models

The problem of insufficient cell material available for DSRT can be solved by using miniaturized platforms based on technologies alternative to microtiter plates. There are numerous studies demonstrating utilization of such platforms for screening of live mammalian cells, however few of them are focused on screening of patient-derived cells with a goal of defining appropriate therapy for cancer patients.[67,84–87] Some examples of such studies are given below and summarized in Table 1.

Pak et al. developed a microfluidic-*cis*-coculture (MicroC3) chip to investigate drug response of primary $CD138^+$ multiple myeloma (MM) cells isolated from 17 patients in co-culture with $CD138^-$ tumor-companion mononuclear cells isolated from the same patient. Anticancer drug bortezomib was used to evaluate the system.[67,84] MicroC3 chip consists of an array of separated culturing units containing a central well and two side chambers connected to the central well through channels that allow for exchange of paracrine signals but prevent cell-to-cell interaction. This system is operated by passive pumping and needs only a pipette to operate. About 7500 tumor cells in 5 µL medium were seeded through an inlet port of the central well, while healthy cells were introduced through an inlet of the side chambers. The authors used statistical methods to segregate patients into clinically responsive and nonresponsive ones based on DSRT. Using a co-culture system, all 17 patients were segregated correctly judging by their clinical response. Using a monoculture system, patients could be segregated only with 65% accuracy showing that co-culture of tumor cells with healthy cells from their microenvironment gives more accurate estimation of clinical response.[67] Ma et al. developed a microgap plate (MGP), a 96-well-formatted microfluidic plate with built-in micro-gaps (**Figure 3**a) that enables trapping and screening of only 1000 cells per experiment.[88] Cells are in contact with large medium volumes and could be washed without losing cell content even in case of suspension cells. The authors evaluated the device on cancer cell lines as well as on primary cells from breast cancer patients and demonstrated good concordance between dose-response obtained on their platform and

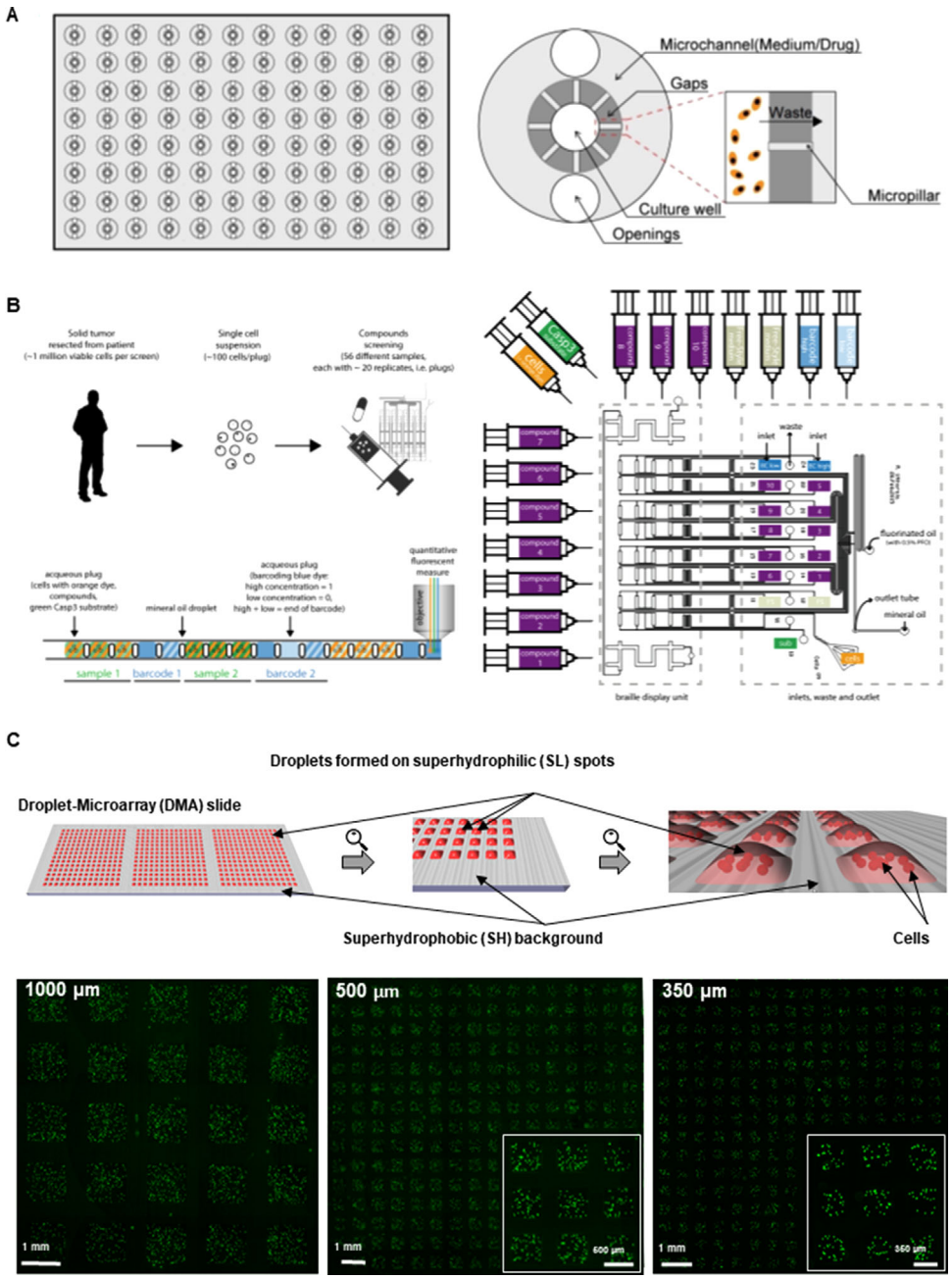

Figure 3. Miniaturized 2D cell culture systems. a) Schematic representation of microgap plate (MGP). Reproduced with permission.[86] Copyright 2014, RSC. b) Microfluidic based chip for rapid identification of optimal drug combinations. Workflow for patient samples (top left panel). Barcoding system to distinguish between different drug combinations (bottom right panel). Design of the chip. Sixteen syringes with aqueous samples are connected to the inlets in the microfluidics chip via tubing (ten with compounds, two with medium to generate single drug and control samples, two for barcoding, one for the cell suspension, one with Caspase 3 substrate to detect apoptosis). Other two inlets in the microfluidics chip are used for carrier oil (FC-40) and mineral oil (right panel).). Reproduced with permission.[87] Copyright 2018, Springer Nature. c) Schematic representation of Droplet-Microarray platform (top panel). Microscopy images of arrays of cells formed on Droplet-Microarray with spot sizes of 1, 0.5, and 0.35 mm. Reproduced with permission.[92] Copyright 2016, SAGE Publications.

conventional 96-well plates.[88] Fujii et al. fabricated a microfluidic chip containing eight parallel channels.[85] The SH-10-TC stomach cancer cells were introduced into the chip in the amount of 10 000 cells in 10 µL. A gradient of 5-fluorouracil was generated by mixing two dilutions containing 0.1–9.8 mg mL^{-1} of compound, achieving eight different concentrations in parallel channels and using 1250 cells per concentration. Viability of cells was estimated microscopically after 24 h by staining dead cells with EthD-1. The authors observed clear concentration-dependent effect of drugs on primary cells using their system.

Correlation with clinical outcome was not assessed.[85] Eduati et al. developed a miniaturized system for combinatorial screening of anticancer therapeutics based on a droplet microfluidic platform (Figure 3b). Droplet microfluidics is based on the formation of an emulsion of aqueous droplets in oil phase in the microfluidic channel.[89] Such droplets are capable of trapping live cells and can serve as a mini-reservoir for screening applications.[89] The authors built a system capable of testing ten drugs in all the pairwise combinations, in total 1100 samples, in droplets containing single cells using 100 cells per group of droplets (plugs) with the same drug combination and 20 plugs per each drug combination (Figure 3b). To set apart all the combinations, they came up with a sequential barcoding system dividing each group of droplets inside a microfluidic chamber with sequences of droplets with binary (high/low) concentrations of the blue fluorescent dye (Figure 3b). For estimation of drug toxicity, a rhodamine 110 (green fluorescent dye) conjugated substrate of Caspase-3, an early marker of apoptosis, was encapsulated in each droplet with cells. To be able to verify the dilutions of all reagents, Alexa Fluor 594 (orange-fluorescent dye) was added to each droplet (Figure 3b, bottom left panel). Multiple droplets for each drug combination were generated on a chip following by barcoding of each droplet with fluorescent dye. Afterward droplets containing cells were incubated in a gas permeable tubing for 24 h. After incubation period, all the droplets were passed through a detection system with three different excitation lasers (375, 488, and 561 nm) to detect 1) barcodes defining which drug composition is in the droplets, 2) Alexa Fluor 594 to monitor reagent dilution, and 3) Caspase-3 activity to estimate level of apoptosis. Using this system, authors could successfully perform a screen with all 1100 combinations of anticancer compounds using in total only 1 million cells obtained from four pancreatic cancer patients demonstrating distinct responses of individual patients.[87]

In our laboratory, we have developed a universal miniaturized platform for screening applications based on arrays of hydrophilic spots created on a superhydrophobic background (Figure 3c).[90–92] Hydrophilic spots have usually round or square shape and their diameter or side length can vary from 300 to 1000 µm. Due to the extreme differences in wettability between hydrophilic and superhydrophobic areas, applying aqueous solution onto such hydrophilic-superhydrophobic patterned surface leads to spontaneous formation of arrays of homogeneous nanoliter-sized droplets, each of which can serve as a nanoreservoir for cell experiments. The size of culturing volumes ranges from 5 to 200 nL and can be adjusted to a particular application (Figure 3c). Such Droplet-Microarrays are compatible with standard laboratory equipment including automated microscopes, fluorescence scanners, and non-contact liquid dispensers for compounds and cells. It is compatible with microscopy-based read-out and related procedures including staining, fixation, and immunofluorescence protocols. We have demonstrated the use of Droplet-Microarrays for multiple screening application of adherent and suspension cells, as well as for 3D cell culture.[91,93] In a recent study, we performed a screen of nine compounds on only 100 patient-derived chronic lymphocytic leukemia (CLL) cells per a 100 nL droplet. Dose responses obtained on the Droplet-Microarray platform using microscopy-based read-out were in good agreement with results obtained in 384-well plates using both microscopy-based estimation of viability and CellTiter Glo assay (manuscript in under review).

In majority of studies described above, the platforms utilize microfluidic principle. The main advantage of such systems compared to microtiter plates is using on average about an order of magnitude less cells and reagents for screening. In addition, microfluidic systems allow for creating perfusion systems for long cultivation times and possibility for convenient media exchange. The drawback of described setups is still limited (up to 96) throughput of compounds that can be analyzed. Systems based on droplet microfluidic principle open the possibilities to go down to single cell and pico- to nanoliter volumes. However, such systems can be limited in their throughput, or have to be used with complex barcoding system. Open 2D array format platforms such as Droplet-Microarray allow for using three orders of magnitude less cells and compounds, are designed for short-term (2-3 days) cultivation time, and enable scaling up the throughput of compounds up to thousands per small area of standard microscopic glass slide. All types of described miniaturized systems carry different properties and can be applied for different application requirements.

The main purposes of systems, alternative to the state-of-the-art microtiter plates for screening of patient-derived cells in 2D models are 1) miniaturization, which enables the use of an order of magnitude less cells and reagents for screenings; 2) possibility to create flexible perfusion systems for long cultivation times; and 3) higher throughput, which enables screenings of large libraries of drugs. Majority of the described platforms are based on the microfluidic principle, and enable miniaturization and creation of customized and flexible culturing solutions; however, such systems are not always compatible with screening of large drug libraries. Open 2D array format platforms enable miniaturization and high throughput. The choice of a platform for the test should be defined by the aim of the experiment.

8. DSRT on 3D Cell Culture Models

In the past two decades, 3D cell culture has been picking up as a model that more closely represents in vivo situation. Tumor spheroids and organoids, also called organotypic multicellular spheroids, are examples of most commonly used 3D cell models in oncology. Tumor spheroids are tight spherical aggregates of tumor cells that are derived from self-organization of single cells, which can be represented by cancer cell lines or patient-derived cancer cells.[94] Tumor organoids, in oppose to spheroids, are formed by mechanical or enzymatic disintegration of the original tumor tissue into small fragments followed by culturing these tumor fragments in extracellular matrix network, such as Matrigel.[95]

Tumor spheroids are simple and reproducible in vitro model, which, in comparison with 2D monolayer cell culture, exhibits characteristics of a tumor including in vivo features such as morphology, formation of a hypoxic core, production of extracellular matrix (ECM), cell–cell interaction, and protein, as well as gene expression patterns.[96] It was demonstrated in multiple studies that cancer cells cultured in vitro in 3D versus 2D respond differently to the drug treatment resembling closer in vivo response.[97] Tumor spheroids take only few days to

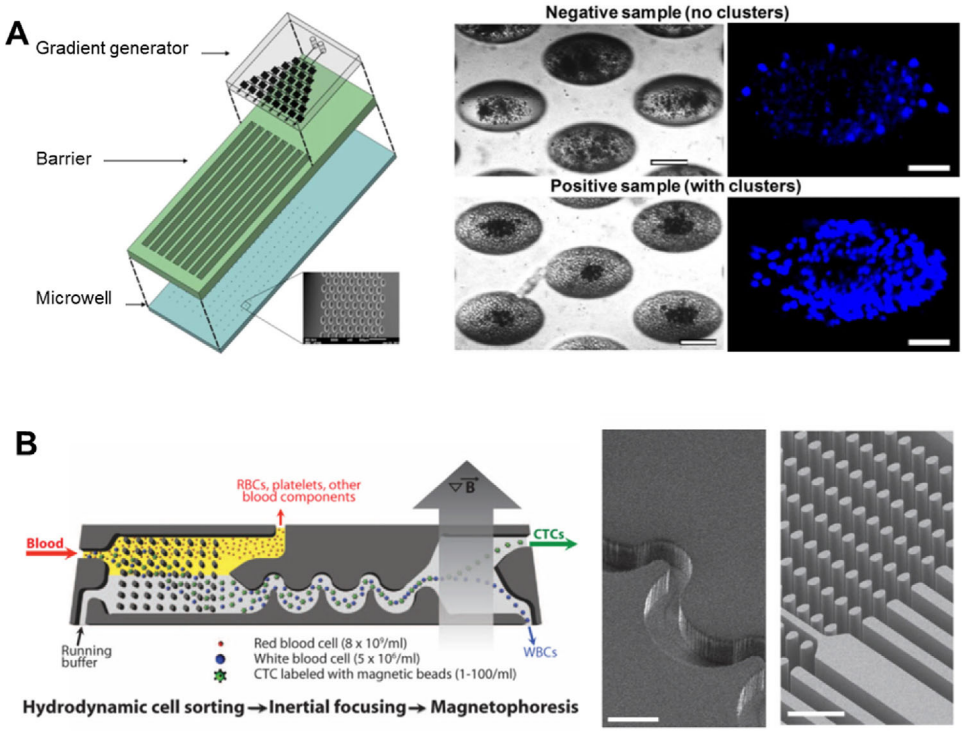

Figure 4. Microfluidic based systems for the isolation and testing of CTCs. a) Three-dimensional layout of a platform for CTC cluster assay (left panel). Representative bright-field images of microwells comprising a negative and positive sample, scale bar, 100 mm, and Hoechst staining of cell clusters, scale bar, 50 mm (right panel). Reproduced with permission.[114] Copyright 2016, The Authors, published by AAAS. b) Schematic representation of CTC-iChip system for isolation of CTC cells from whole blood (left panel). SEM images of post-array (left) and asymmetric focusing units (right). Adapted with permission.[116] Copyright 2013, AAAS.

form and can be a suitable model for DSRT, where spheroids formed from freshly patient-derived cells are tested against a panel of anticancer compounds. Some examples of studies utilizing spheroids for DSRT are presented below and in Table 1.

Jeppesen et al. successfully formed spheroids from cells obtained from 15 of total 18 patients (83% success rate) with colorectal cancer in Petri dishes coated with agarose.[98] Obtained spheroids consisted mostly of epithelial cells with up to 5% of fibroblast contamination and closely represented original tumor in terms of morphology and protein expression patterns (**Figure 5**a). After 3 days of culturing, spheroids were transferred into 96-well plates coated with agarose and introduced to anticancer compounds for another 7 days. Authors observed distinct patterns of dose-response in spheroids obtained from five different donors indicating maintenance of individual drug sensitivity profiles in spheroids during culturing period. Correlation of DSRT results with clinical outcome was not discussed.[98] Hagemann et al. studied formation of spheroids from the PiCa cell line, which was generated from primary carcinoma specimens without enzymatic digestions of single cells.[99,100] Spheroids were grown using two methods—1) the method of hanging droplet, where cell aggregation is promoted by gravitational accumulation of cells in the water–air interface of droplets; and 2) ultra-low attachment plates (ULA), where cells are prevented from attaching to the surface, which leads to cell aggregation. The authors successfully formed spheroids using both methods and demonstrated reduction of spheroid diameter by treating them with cisplatin, 5-fluorouracil (5-FU), or radiation.[99]

Tumor organoids or organotypic multicellular spheroids is an example of 3D cell culture model of a primary tumor tissue obtained from fresh biopsies.[94] Since organoids are derived from tumor fragments, they closely resemble the tissue that they originate from including cell heterogeneity and presence of different cell types. Tumor organoids can be passaged, expanded, and cryopreserved similar to cell lines. They can be generated from fine-needle aspirations or surgically removed tumor within few weeks and used for molecular profiling of a tumor, as well as for individualized therapeutic screening (pharmacotyping).[95,101] There is an increasing number of studies where patient-derived tumor organoids are used for DSRT and other precision medicine studies, some of which are described below and summarized in Table 1.

Jabs et al. compared effects of anticancer drugs on cell lines derived from primary ovarian tumor cells cultured either in 2D monolayers or in 3D organoids.[102] Organoids were formed for 10 days of culturing cells in Matrigel. The authors developed a DeathPro pipeline, which is a confocal microscopy-based assay and image processing workflow to simultaneously assess cell death and growth arrest. Cells were incubated with Hoechst and propidium iodide, and both the total number of cells and dead cells were monitored during the cultivation. The authors performed a screen of 22 compounds and their combinations on both 2D and 3D cell models and concluded that

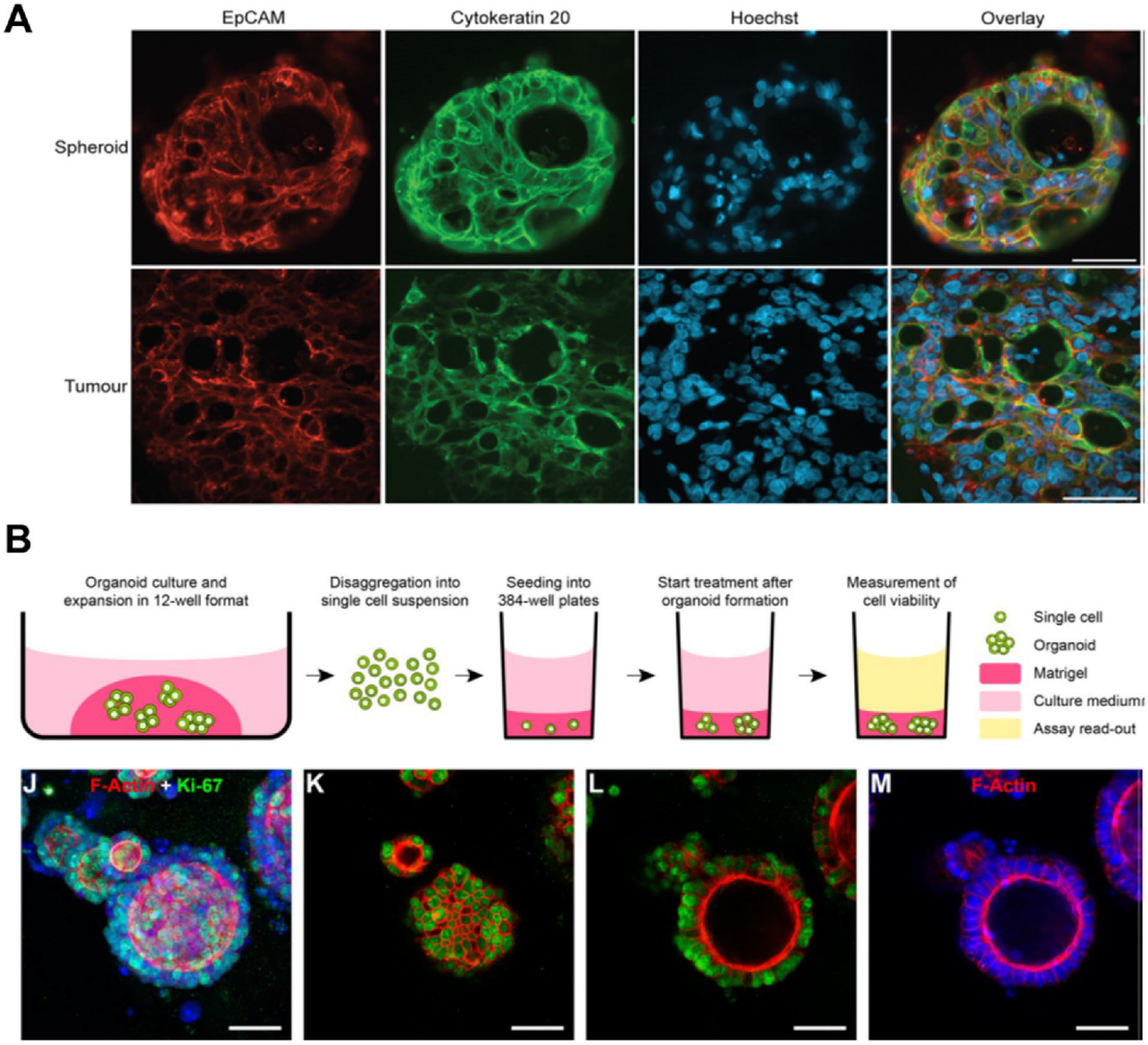

Figure 5. 3D cell cultures established using patient derived material. (a) Immunostaining of spheroids and corresponding tumors from one patient for epithelial cell marker EpCAM (red) and gastrointestinal epithelial marker cytokeratin 20 (green). Nuclei are stained with Hoechst (blue). Size bars = 50 µm. Reproduced under the terms of the CC-BY license.[98] Copyright 2017, The Authors, published by PLoS One. b) Workflow of establishment of patient-derived organoid cultures in 384-well format (top panel). Confocal image analysis of patient-derived organoid cultures in 384-well format stained for F-actin (phalloidin, red), Ki-67 (green), and DAPI (blue) illustrates the maximum intensity projection (J), an optical section of the surface (K), and the DAPI-negative luminal compartment of the canter (L, M) of an organoid structure. Scale bar 50 µm (bottom panel). Reproduced with permission.[103] Copyright 2016, SAGE Publishing.

drug-induced cell death was comparable, while drug-induced growth arrest varied between 2D and 3D cell cultures.[102] Boehnke et al. cultured organoids formed from tumor cells of patients with colon cancer.[103] Organoids were first cultured and expanded in Matrigel droplets in 12-well plates for several weeks till aggregates reached 800 µm in diameter. For the compound screening, organoids were then disintegrated into single-cell suspension and 5000 cells were seeded in 384-well plate in Martrigel (Figure 5b, top panel). After 4 days, organoids were formed (Figure 5b, bottom panel) and introduced to compounds for 3 days. Toxic effect of the anticancer compounds was estimated by measuring ATP content using luminescent based assay. Established workflow was validated for its robustness and reproducibility with organoids established from cells obtained from four different donors.[103] Pauli et al. developed a combinatorial panel, where authors formed organoids from patient-derived tumor cells and compared data from the whole exome sequencing (sequencing of all of the protein-coding genes in a genome, called exome), in vitro drug sensitivity, and in vivo drug sensitivity of xenografts derived from the formed organoids.[104] The authors

collected overall 145 specimens, representing 18 different tumor types. Organoids were successfully formed from 56 specimens by culturing and passaging freshly isolated tumor fragments in Matrigel. The main aims of that study were to identify optimal therapy based on in vitro and in vivo tests and create a database connecting drug sensitivity to genetic background. In vitro and in vivo drug sensitivity tests were performed on organoids derived from four patients by plating 1000–3000 cells in Matrigel in 96-well plate and estimating drug effect by using luminescent-based CellTiter Glo assay. Optimal therapy identified based on the results of in vitro tests was implemented for two patients. In both cases the recommended therapy performed better compared to the existing standard therapy, showing the importance of such tests in predicting response in patients.[104] Vlachogiannis et al. established a biobank of patient-derived organoids (PDOs) from metastatic colorectal and gastroesophageal cancer patients.[105] The authors demonstrated that phenotypic and molecular profiling of established PDOs closely matched the original tumor. They compared ex vivo drug response of PDOs with xenograft mouse models, as well as with clinical response in patients and demonstrated that their methodology can predict clinical response with 100% sensitivity and 93% specificity.[105]

3D cell culture models, such as tumor spheroids and organoids, based on patient-derived cells closer represent in vivo situation in comparison to 2D cell models, therefore such models are more physiologically relevant and predictive of drug response in patients. Establishing such models, however, associated with some practical hurdles and very often takes days or weeks to be established. In context of clinical test, the advantages of physiological relevance have to be balanced with cost and time of the test.

9. Miniaturized Systems for DSRT on 3D Cell Culture Models

All previously described systems for fabrication and screening of patient-derived 3D cell culture models were performed in Petri dishes and microtiter plates format. This format is associated with high consumption of reagents and especially cells, which is critical when working with limited patient-derived material. Miniaturized microfluidic-based platforms for fabrication and screening of patient-derived 3D cell culture models are addressing these problems. In addition to miniaturization of assays, implementation of microfluidic-based systems allows for the formation of more complex tumor-on-a-chip systems that closely represent in vivo situation. In such systems, it is possible to combine 3D cultures from different organs, co-culture multiple cell types, creating perfusion systems, and drug gradients. These types of complex tumor-on-a-chip systems are reviewed elsewhere.[106,107] Majority of such systems are developed and evaluated on cancer cell lines and represent great models for studying fundamental biological processes. In addition, tumor-on-a-chip systems can be very useful in drug discovery pipeline, for example, for toxicity assessment of new drug candidates. However, these systems are rather complex for utilization in clinics, where fast, simple, and miniaturized systems are preferable. There are several studies describing simple microfluidic-based systems for performing DSRT on patient-derived spheroids and organoids.

In the work of Ruppen et al., a microfluidic chip containing multiple independent channels and micro-wells was created (**Figure 6**a).[107] The system was evaluated with spheroids derived from patients with non-small cell lung adenocarcinoma as monoculture and co-culture with pericytes isolated from the same patient. Spheroids were formed from 5000 cells (Figure 6a, right panel), incubated with cisplatin for 2 days, followed by toxicity quantification by the measurement of caspase-3/7 activity. The results showed that co-cultured spheroids were more resistant to the therapy compared to monoculture spheroids.[107] Mazzocchi et al. developed a microfluidic device consisting of multiple independent channels opening into a culturing well in the middle (Figure 6b).[108] Tumor cells from fresh tumor biopsies from two mesothelioma patients were cultured in these wells incorporated in ECM-mimicking HA/gelatin-based hydrogel. Authors demonstrated formation of organoids in their system, which maintained mesothelioma phenotype for extensive period of time. Drugs were introduced through perfusion channels and their effect was estimated by live/dead staining using Calcein/ethidium homodimer-1 followed by confocal microscopy. The results demonstrated good agreement between the sensitivity of organoids to anticancer drugs estimated in the microfluidic system and the clinical response of the patients.[108] In another example, a device containing multiple independent rows of five connected chambers for culturing cells and creating a gradient of compounds along those chambers was developed.[109] Three-dimensional cell cultures were formed in chambers in Cultrex BME (as a substitute for extracellular matrix) from tumor and stroma cells of fresh lung cancer tissues obtained from eight patients with lung cancer. Obtained 3D cell cultures were tested against two anticancer drugs and their combinations. Percentage of apoptotic cells was defined by apoptotic-specific staining followed by confocal microscopy. Obtained sensitivities profiles were in good agreement with profiles obtained in 96-well plates using MTT assay.[109]

The use of 3D cell cultures derived from patient materials for identification of personalized anticancer therapy is a relatively recent approach. There are few studies showing positive correlation between DSRT performed on tumor spheroid/organoid cultures and short-term clinical response of the patients.[108] However, there is no study yet comparing long-term survival rate of patients, treated with therapy recommended based on results of DSRT and treated with conventional therapy.[101,110]

Circulating tumor cells (CTC) are cells that detach from original solid tumor and enter blood circulation. They are very important for monitoring drug sensitivity of a tumor because of two reasons. First, they represent a population of metastatic cells that cause 90% of cancer deaths.[111] Second, blood sampling is non-invasive and can be repeated during and after the treatment to monitor tumor clone evolution and development of drug resistance. The main challenge in studying CTCs is that there are only 1–100 cells mL^{-1} of blood present in patients with metastatic disease. There are studies describing isolation of CTCs from the blood using microfluidic-based systems.[112] The problem of screening these cells, however, persists, because only few hundred to several thousand cells can be obtained from a blood sample. In order to be able to perform comprehensive screening of such small cell numbers, highly miniaturized platforms have to be used. Bithi et al., for example, developed a

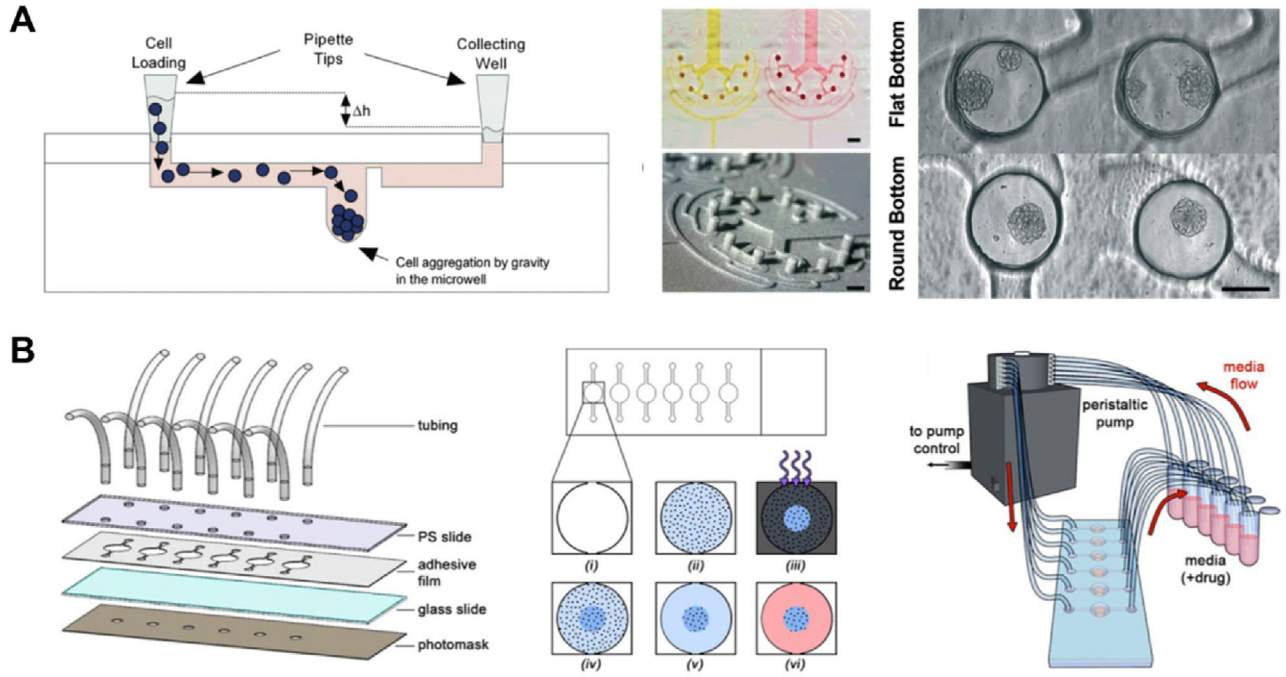

Figure 6. Miniaturized 3D cell cultures designed for DSRT experiments. a) Perfused microfluidic system for forming and screening of 3D tumor spheroids developed by Ruppen et al.[107] Cells are loaded in the system using hydrostatic pressure and are trapped in the microwells by gravity (left panel). Top-view picture of two microfluidic channels filled with red and yellow food dyes (middle panel top). Image of an epoxy mold with rounded micropillars, representing the negative of the final channels. Scale bar 1200 µm. (middle panel bottom). Representative images of spheroids formed in microwells after 3 days in culture. Scale bar corresponds to 250 µm (left panel). Reproduced with permission.[107] Copyright 2015, RSC. b) Microfluidic device for forming and screening of organoids developed by Mazzocchi et al.[108] (left panel). In situ organoid patterning technique (middle panel): a microfluidic chamber (i) is filled with a mixture (blue) containing hydrogel precursors, photoinitiator, and patient-derived tumor cells (ii), and then illuminated with UV light through a photomask (gray) (iii). Exposed precursor is crosslinked into a hydrogel (dark blue), detaining cells within the region (iv), and non-crosslinked gel is flushed form the chamber with clean PBS from the chamber (v). Finally, PBS is replaced with DMEM (red) (vi) for incubation (middle panel). The total measurement set-up, featuring a low-volume, closed loop fluidic circuit for each organoid facilitated by a computer-controlled peristaltic pump (right panel). Reproduced with permission.[108] Copyright 2018, Springer Nature.

pipette-based microfluidic cell isolation (MCI) chip, which allows for the formation of an array of trapped static droplets, containing single cells or cell clusters, from 10 µL of cell suspension containing from 10 to 100 cells. Cells can be introduced to compounds and screened using microscopy-based read-out directly on the chip. The proof-of-concept study was performed on MCF-7 breast cancer cell line and did not involve patient-derived CTC.[113] Khoo et al. demonstrated an approach to test patient-derived CTCs from whole blood with anticancer compounds without cell pre-isolation within 2 weeks.[114] The authors developed a microfluidic device containing eight channels (for drug gradient formation), each of which contained an array of oval cavities with 100 µm major axis (**Figure 4**a, left panel). Nucleated cell fraction from whole blood containing both CTCs and WBCs were seeded into the channels. Ratio of CTCs to WBCs was from 1/100 to 1/1000, which translated in approximately one CTC per cavity (Figure 4a, right panel). Since CTCs are rapidly proliferating and forming 3D cell clusters in vitro, while WBCs do not, cluster formation was an indication of presence of CTCs in blood samples. Therefore, cluster formation is an indication of presence of malignant tumor in the body and correlates with bad prognosis for the patient. As expected, there was no cluster formation observed in blood samples from healthy donors (Figure 4a, right panel). Drug treatment was performed using only samples positive for cluster formation. Viability of cells in clusters was estimated using live/dead staining and microscopy. Clear dose-responses of CTC clusters to anticancer compounds were obtained by using this system.[114]

Another possibility to perform screening on isolated CTCs is to expand them in vitro and establish a stable long-term cell culture.[115] In the work of Ozkumur et al., authors used CTC-iChip,[116] a microfluidic-based platform to separate CTCs from the whole blood. Working principle of CTC-iChip is based either on using positive selection, in which CTCs are isolated on magnetic beads with anti-EpCAM antibodies, or negative selection, in which the blood is depleted of leukocytes by using magnetic beads with antibodies specific to common leukocyte antigen CD45 and the granulocyte marker CD15 (Figure 4b).[116] In the work of Yu et al., authors used this platform to isolate cells from patients with metastatic breast cancer and successfully established cell lines from six patients. These cell lines were cultured in non-adherent in vitro conditions forming 3D cell aggregates. The authors performed sequencing analysis in parallel with DSRT with a goal to find correlations in drug sensitivity and genetic context of each individual patient.[117]

Miniaturized systems for DSRT on 3D cell culture models are mostly based on microfluidic principle and along with miniaturization and decreasing the number of cells needed for the test,

allow precise spatial and geometric control of culturing vessels, which is very important for the formation of 3D aggregates. Such platforms also give possibility to form more complex perfusion tumor-on-a-chip systems co-culturing several cell types, which can make tests on patient-derived cells more predictive. At the same time, most of these solutions are rather low throughput and not compatible with screening of large drug libraries. Therefore, the choice of the screening platform should be defined by the type (type of cancer, number of drugs) and purpose of the test.

10. Acceptance of Individual Prognostic Tests by Healthcare System

Precision medicine in oncology today includes molecular profiling and DSRT. Molecular profiling includes either targeted sequencing for known oncogenes or more complex profiling covering genome, epigenome, transcriptome, and metabolome analysis.

Targeted sequencing for known oncogenes is applied in clinics to identify if patient carries known mutations in these genes and will respond to a certain targeted therapy. There are several panels of genes that were approved by FDA for targeted sequencing.[118,119] Genome profiling is performed by multiple companies, including Foundation Medicine, Caris Life Science, Guardant Health, GenomeDx Biosciences, Intermountain Healthcare, Genomic Health, Trovagene, Varientyx, Invitae, Paradigm, and has a cost of several thousand U.S. dollars. Depending on diagnosis, the cost of such test may be covered by some medical insurances, including Medicare in the United States.[119]

The aim of more complex molecular profiling, covering genomic, epigenomic, proteomic, and metabolomics analysis, is to identify known changes in those profiles that can correlate with sensitivity of a tumor to a particular therapy. Mostly such profiling is still performed for research purposes. It is not part of clinical routine but can be performed privately by a patient as an additional information that can help to find suitable treatment.

DSRT is used in addition to molecular profiling to test the sensitivity of patient derived cancer cells to anticancer compounds in vitro. There are a number of studies analyzing previously published work on DSRT and its ability to predict clinical response in patients. Literature published during the past four decades on DSRT performed on >15 000 tumor patients demonstrate that such tests can predict short-term sensitivity of tumor to a drug with accuracy of 50–80%, and short-term resistance with accuracy of 80–100%.[73,120] In other words, if cells are sensitive to a drug in vitro, the tumor will be sensitive to this drug in vivo with probability of 50–80%; and if cells are resistant to a drug in vitro, the tumor will be resistant to this drug in vivo with probability of 80–100%. There are several randomized clinical studies investigating if life expectancy of patients treated with drugs recommended by these tests was different compared to life expectancy of patients that were treated with conventionally prescribed therapy. About 25–35% of clinical reports stated improvement of patient survival by using recommendations based on DSRT results.[23,121] Based on this statistics, DSRT is not overall recommended as a predictive test by the American Society of Clinical Oncology.[122] In clinical practice, DSRT is offered by some private companies, such as TherapySelect, ChemoFx assay, and CorrectChemo assay. The cost of the test can reach several thousand U.S. dollars and usually is not covered by insurance companies. The majority of DSRTs are performed in research laboratories inside hospitals and are used for research purposes being utilized as a recommendation for drug therapy only for late stage cancer patients, which did not respond to standard therapy.

At the moment, the only personalized test that is accepted in clinics and covered by insurance is targeted sequencing, which is recommended by the practitioner for certain types of cancer. Full molecular profiling and DSRT are not mainstream accepted prognostic tests and considered depending on the situation of the patient. As a rule, usually such tests are not covered by medical insurance.

11. Toward Acceptance of DSRT as a Prognostic Test in Clinics

DSRT can significantly improve selection of individualized therapies for each patient. However, at the moment it is not recommended as a predictive test and not applied in clinics as a common practice. There are a number of issues discussed below with how DSRT is performed at the moment, which hinders it from being established in clinics.

11.1. Only Few Drugs are Tested in DSRT

At the moment on average about ten drugs (with exception of blood cancers) are tested in DSRT, which leaves out compounds that might be effective against a tested tumor.[23] In addition, it is important to test combinations of drugs in DSRT, since combinatorial therapies are proven to be more effective compared to drugs applied as mono therapy.[123] In order to achieve this, the protocols and platforms utilized for DSRT should be compatible with high throughput and be highly miniaturized. Commonly used microtiter plates have to be substituted by Lab-on-a-Chip technologies that enable parallel screening of hundreds to thousands of different compound combinations on a single patient sample in nanoliter (instead of microliter) volumes.

11.2. DSRT is Performed Only Once Before the Therapy

In majority of published studies, DSRT is performed once before the therapy is introduced to a patient. In vitro test can predict a short-term response of a tumor to a drug, because cells that are in majority at the moment in the tumor are tested. However, due to constant clonal evolution within a tumur, some cancer cells that did not respond to the first therapy, might proliferate further and cause a relapse of a disease. Multiple DSRTs before and after the therapy will allow to spot drug sensitivity of new emerging cancer cell populations. This will help to adjust the therapy throughout the treatment in order to completely eliminate cancer cells from the body. For this, taking the multiple biopsies from patients before, during, and after the therapy, followed by performing DSRT on cells isolated from these biopsies, should

be introduce to clinical practice. One important prerequisite for this practice to be adopted in clinics is low cost of DSRT test, which can be achieved by highly miniaturizing the test using Lab-on-a-Chip technologies.

11.3. DSRT is Performed Only on Cells from Original Tumor

Usually DSRT is performed on cells from original tumor. However, it is critical to test cancer cells from metastatic locations as well, because they represent the most dangerous cell population causing 90% of death and might differ from the cells in original tumor in their response to a therapy.[111] In addition, it is important to perform DSRT in parallel on healthy cells in order to define individual therapeutic index of drugs. Knowing individual therapeutic index of a drug for each patient is extremely important not only for defining the drug that is efficient against individual tumor and not toxic to normal cells, but also for identification of suitable concentration of a drug for individual patient.[81] In order to have an overview of all individual characteristics of tumor and healthy cells for each patient, it is important to obtain multiple samples of cells from different sites from the patient, including cancer cells from the site of original tumor, metastatic sites, CTCs, as well as healthy cells followed by performing DSRT on each of these cell types. Considering the cost of the test and that only minute number of cells can be obtained from CTCs or metastatic site, DSRT test has to be highly miniaturized.

11.4. DSRT Requires Large Number of Cells

At the moment, DSRT is performed in 384- or even 96-well plates, requiring from 5000 to 200 000 cells per well. This is the reason why DSRT is only performed when relatively large number of cells is available as in case of blood cancer or surgically removed solid tumors. When cells are obtained with needle biopsy from original tumor or metastatic sites, there is not enough cell material available for DSRT. Using alternative miniaturized technologies that enable tests on low cell numbers in small volumes can be a solution. For example, with 500 000 cells on average obtained from needle biopsy, only 50 experiments can be performed in 384-well plate (10 000 cells per well) and five in 96-well plate (100 000 cells per well). By using only 100 cells per well, it would be possible to perform 5000 experiments per each patient. Performing large screenings will increase number of drugs and their combinations that can be tested for one patient, giving wider selection for the therapy. In order to achieve it, highly miniaturized platforms that enable parallel screening of minute number of cells in small (nanoliter) volumes have to be adopted in clinical laboratories.

11.5. Most DSRTs are Performed on 2D Cell Cultures

Three-dimensional cell cultures are known to closer represent in vivo cell environment compared to 2D cell models, which makes them more suitable models for predicting drug responses in patients. There is a number of studies using 3D cell cultures for DSRT.[102–104] However, most of the studies in DSRT were performed using 2D cell culture models. The reasons for that are that 3D cell models are more difficult to establish and analyse, and in many cases, they require even more cells compared to 2D cell culture. Platforms and methodologies that enable easy formation and analysis of homogeneous spheroids and organoids in single-per-well manner with low input of cells are absolutely needed in order to establish DSRT on 3D cell culture as a test in clinics.

11.6. DSRT is Not Standardized

At the moment, DSRT is performed mostly in the research laboratories that work together with hospitals. Each laboratory uses different experimental setup. Clinics that do not have excess to such research facilities, do not have possibility to perform DSRT. Companies that offer DSRT as a service are scarce and follow different experimental setups as well. Standardization of DSRT by defining standard and optimal formats (2D or 3D) and experimental setup (platform, treatment time, read-out assay) is critical. In order to be applied in clinics, DSRT should be robust, simple, performed on minute amounts of cell material, and inexpensive.

11.7. Not Sufficient Evaluation of DSRT in Clinics

According to the published randomized clinical studies focusing on predictability of DSRT for long-term survival of patients, only about 25–35% claim positive influence of DSRT on defining suitable therapy for patients.[23,121] However, all the issues stated above about how DSRTs are performed contribute to this poor positive correlation rate. There is a clear need to address these issues and perform more clinical studies investigating whether choosing therapy for individual patients based on results of DSRT has an influence on short- and long-term response of patients to the therapy.[51]

There are a number of issues that need to be addressed in order for DSRT to be accepted as a prognostic test for defining anticancer treatment in clinics. The DSRT technology should become compatible with: 1) low cell numbers; 2) high throughput; 3) physiologically relevant cell models; 4) multiple testing before and after the therapy. 5) DSRT methods should be used on cells originating from both original tumour and metastasis. The test should be 6) standardized and 7) more clinical studies should demonstrate the relevance of DSRT. In order to achieve these goals, the protocols and platforms utilized for DSRT should be compatible with high throughput, be highly miniaturized and affordable. Commonly used microtiter plates have to be substituted by technologies that enable parallel screening of hundreds to thousands of different compound combinations on a single patient sample in nanoliter (instead of microliter) volumes. Taking multiple biopsies from patients before, during, and after the therapy, as well as from different sites, including original tumor, metastatic sites, CTCs, as well as healthy cells, followed by performing DSRT on cells isolated from these biopsies, should be introduced as a routine in clinical practice. It will take some time till such tests will be optimized and standardized for clinical use.

First clinical studies need to be performed to collect large data sets and demonstrate that DSRT has a predictive value for different types of cancer. Therefore, it is important to investigate different cancer types and stages, to test cells from different sites of cancer and multiple times during treatment, and to monitor short-term response, as well as long-term survival rates of patients.

12. Concluding Remarks

Based on numerous DSRT studies and corresponding published rates of prediction of short-term clinical response in patients, it appears that DSRT is an important method that can help in prediction of short- and eventually long-term responses of individual cancer patients to a therapy. Using results obtained from DSRT in combination with molecular profiling data can help practitioners to have a more comprehensive picture of nature of individual cancer, and to select a suitable therapy for each patient. There are some challenges discussed in this review that have to be addressed in order for DSRT to be established as a routine test in clinics. Considering high level of heterogeneity of cancer and the fact that majority of patients do not respond to standard therapies, it is absolutely necessary to personalize cancer treatment and test tumor cells from individual patients for sensitivity and resistance against anticancer drugs before the treatment. Introducing DSRT as a routine test for estimation of sensitivity and resistance of tumour cells to a therapy before, during, and after the therapy, in addition testing healthy cells of a patient for adverse effects, can increase efficiency of a therapy, reduce side effects, and lead to longer survival rates.

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Conflict of Interest

The authors declare no conflict of interest.

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