Cell-Based Microarrays for In Vitro Toxicology

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Abstract

DNA/RNA and protein microarrays have proven their outstanding bioanalytical performance throughout the past decades, given the unprecedented level of parallelization by which molecular recognition assays can be performed and analyzed. Cell microarrays (CMAs) make use of similar construction principles. They are applied to profile a given cell population with respect to the expression of specific molecular markers and also to measure functional cell responses to drugs and chemicals. This review focuses on the use of cell-based microarrays for assessing the cytotoxicity of drugs, toxins, or chemicals in general. It also summarizes CMA construction principles with respect to the cell types that are used for such microarrays, the readout parameters to assess toxicity, and the various formats that have been established and applied. The review ends with a critical comparison of CMAs and well-established microtiter plate (MTP) approaches.

1. INTRODUCTION

In vitro: experiments with living cells but performed outside the complexity of a living multicellular organism

Cell-based assay (CBA): bioanalytical assay based on the response of living cells to a given chemical, biological, or physical stimulus

In vivo: experiments performed inside a living multicellular organism

HeLa cells: first human cell line successfully propagated in the laboratory

Primary cultured cells: animal cells used for in vitro experiments immediately after isolation from the donor organism without further multiplication

Stem cells: animal cells with the ability to proliferate and differentiate into all or a subset of all cell phenotypes

Assessing the toxicology of chemicals has been important since we became aware of the potential toxicity of chemicals, gained more knowledge on the harmfulness of compounds, and started being adversely affected. The demand for toxicity testing has risen continuously throughout the years, and testing was predominantly based on animal experiments in the past. Starting slowly in the 1960s, concerns regarding animal testing were growing, for ethical reasons, issues of animal welfare, and also with respect to cost and increasing legal requirements. The 3R concept by Russell & Burch (1) reflects this growing awareness by, for the first time, offering detailed suggestions as to how to reduce, refine or replace [the Three Rs (3Rs) concept] animal experiments in general but in particular for toxicology assessment. An alternative strategy for unraveling the potential toxicity of a given chemical, which was already provided in the original work by Russell and Burch, is the use of cultured cells for in vitro experiments. Cell culture experiments, also called cell-based assays (CBAs), are considered an important intermediate between complex testing in living animals and simple assays in molecularly well-defined binary or ternary systems (2). They provide a significantly improved reproducibility and give the experimentalist much more control over the detailed experimental conditions compared to animal testing. However, CBAs will never be capable of replacing in vivo experiments entirely, as they are missing the interplay between the various organs and tissues of a multicellular organism including organ-specific metabolization, which may contribute significantly to a compound's cytotoxic profile.

When the 3Rs concept was published, experimental techniques to isolate and culture mammalian cells in a laboratory environment were still in their infancy. The first culture of human cells (HeLa cells) had been established just 10 years before in 1951 (3). It was not until the 1980s that cell culture experiments began to substantially contribute to toxicology assessment (4). Cell cultures from almost any mammalian tissue, most often derived from tumors, are now available as primary cultures directly from the donor organism or as established cell lines from (commercial) cell depositories. With these different cell culture models, organ- or tissue-specific aspects of toxicity are addressable. Besides primary cultured cells, which most resemble cells in the in vivo situation, and cell lines with their infinite life span, two more classes of animal cells have become important for in vitro toxicology: (*a*) engineered cell lines that are manipulated by genetically encoded reporter systems to indicate any onset of cytotoxicty with high sensitivity, and (*b*) stem cells potentially serving as a test system for developmental toxicity. Section 2 summarizes the various cell species that are used for in vitro toxicology studies.

As indicated in **Figure 1**, a proper and well-suited cell culture model is just one prerequisite for a meaningful assessment of in vitro toxicology. It also takes sensitive indicators and readout approaches to measure the viability of the cells upon exposure to a given chemical in quantitative terms. The available assays have been categorized in label-based and label-free techniques, both of which are summarized in Section 3.

The most widespread cell viability assays make use of colorimetric readouts of cell supernatants and are routinely applied in microtiter plate (MTP) formats that allow for 96, 384, or even 1,536 assays in parallel. The liquid volume per well is on the order of only a few microliters, limiting any attempt to further increase parallelization and throughput. However, throughput has become more and more important for toxicology screening. One reason is the legal requirement to perform biosafety tests for an enormous amount of existing chemicals, as has been enforced, for instance, in the European Union by the REACH regulation in 2007. REACH imposed enormous pressure on developing high-throughput alternatives to animal testing (5). Moreover, there is a growing demand for in-depth analysis of toxicity with respect to organ-specific, sometimes even tissue-specific, impacts of questionable chemicals. Both called for even higher throughput than

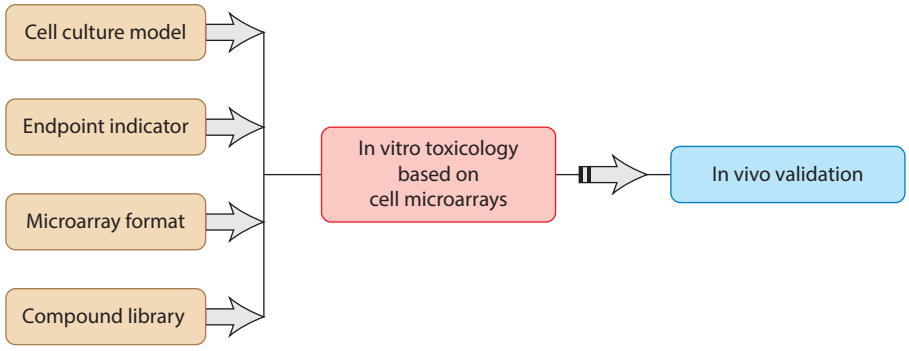

Figure 1

Input parameters to design in vitro toxicology studies based on cell microarrays.

available by MTP formats, fostering the development of cell microarrays (CMAs). CMAs have the potential to overcome the current limitations of parallelization and improve throughput of toxicology screening campaigns with respect to the amount of compounds undergoing testing or the cell types simultaneously included in a screening campaign. Section 4 discusses the available CMA formats and their individual merits and limitations, in particular with respect to toxicology assessment. Section 5 summarizes two examples of successful applications of CMAs for in vitro toxicology studies, providing a state-of-the-art perspective. Section 6 critically evaluates the current status of CMAs with respect to the established MTP formats, which are still the golden standard.

2. CELL CULTURE MODELS FOR TOXICITY ASSESSMENT

The cells that are used as sensors in cell-based toxicology assessment are the most critical and delicate components of the assay, as they have to be uncompromised by the experimental procedure to provide an unbiased and fair judgment on a compound's toxicity. This may seem trivial but it requires that the entire assay fulfill the rather stringent conditions of a cell culture environment (37°C, physiological pH, isotonic solutions, sufficient oxygenation, sterile environment). The cell type preferred for the assay is determined by the requirements of the assay and the questions to be answered.

2.1. Primary Cultured Cells

Primary cultured cells are the most similar to the corresponding cells in the living body, as they are used directly after isolation from the host organism (6). Thus, these cells provide the most relevant physiological information but are typically sensitive to experimental conditions. Ideally, primary cultured cells will not get subcultured, multiplied, or even frozen before they are used in experiments, mainly because proliferation in an ex vivo environment may lead to cell dedifferentiation and selection of those lineages that are capable of adapting the fastest to the laboratory environment. The necessity to isolate these cells on a regular basis makes the use of primary cells less attractive and more time consuming. As mentioned, they do, nonetheless, most precisely mimic the enzymatic activities and overall physiology of their correspondents in the body. With respect to in vitro toxicology, primary cultured liver cells (hepatocytes) are the most valuable sensor cells. However, freshly isolated hepatocytes start to dedifferentiate significantly within the first 24 h ex vivo, which leaves the window of experimental opportunity rather narrow (4).

2.2. Finite or Immortal Cell Lines

Cell differentiation: maturation of an unspecialized cell within a multicellular organism to a highly specialized cell, e.g., neuron, muscle cell

Finite cell lines result from primary cultured cells after prolonged culture and propagation in an in vitro environment. They originate very often from tumor tissues. These cells are proliferative and metabolically active but show a less differentiated phenotype with changes in the protein/enzyme expression pattern. Unless transformation occurs, intentional or unintentional, the cells have a finite life span, undergo cell senescence after approximately 65 doublings (Hayflick limit), and eventually die. After natural or experimentally induced transformation (e.g., by viruses or carcinogens), the cells lose both their growth limitation and inherent finite life span (4). They do not show any signs of senescent deterioration and become immortal. Immortal cell lines are convenient, easy to grow, and available in unlimited quantities but they only poorly mimic the cells in the body—except for general metabolism and cell structure. When not in use, they get frozen and stored in liquid nitrogen until the next round of (toxicology) screening is due (6). The latter issue is particularly important when genetically modified cell lines are used in CBAs. Once a cell line has received a stable genetic manipulation, it can get multiplied and stored for years so that the cumbersome work of genetic engineering does not have to be repeated and the sensor cells persist. An impressive example for the long-term storage and propagation capacity of mammalian cells is the fact that HeLa cells that were explanted from the human body in 1951 (3) are still the most commonly used cell line in biomedical research.

2.3. Genetically Engineered Cell Lines

Genetically engineered cell lines are used heavily in biomedical research. Often they have been tailored to express a receptor or enzyme of interest with much higher copy number than wild-type cells, qualifying them as suitable model systems to study the details of the enzyme or receptor of interest. For in vitro toxicology purposes, animal cells have been engineered to become reporter cells that express an easy to detect reporter gene product upon encounters with cytotoxic substances. The rationale behind this approach is introducing the genetic information of the reporter gene into the genome of the cell line under the control of a stress-sensitive promoter. Well-known examples of such stress-sensitive promoters regulate the expression of heat shock proteins and/or caspases. The latter are known to mediate apoptosis, a genetically encoded, programmed cell death that is triggered whenever the external or internal conditions become unfavorable for the individual cell. Thus, whenever the cells become stressed, the stress-sensitive promoter is activated, leading to the expression of the reporter gene, which is most often a fluorescent protein such as green fluorescent protein (GFP) or enhanced green fluorescent protein (EGFP) (7, 8) or an enzyme that is readily detected colorimetrically. Reporter cell lines have been successfully applied to detect the cytotoxic response of animal or human cells to nanomaterials (9), skin irritants (10), or heavy metals (7).

2.4. Stem Cells

With respect to the cell models that can be used for toxicology screening, stem cells play an important and very special role (11). These cells will not only report on the bare cytotoxicity of a given compound but they will also indicate any impact on cell differentiation if differentiation is addressed in parallel. As such, stem cells in combination with a well-defined differentiation protocol constitute the most well-suited model system for studying developmental toxicity. Moreover, these cells can be differentiated into different phenotypes starting from adipocytes (fat cells), neurons, hepatocytes, or cardiomyocytes, which paves way for assessing a tissue-specific toxicity profile for various human tissues starting from a single population of human (adult) stem cells (11).

3. ENDPOINT INDICATORS IN CELL-BASED TOXICITY STUDIES

Assessment of toxicity requires quantifying the number of viable or dead cells within a given cell population after a predefined exposure to the compounds under examination. So it is the overall rationale of all existing viability/cytotoxicity assays to determine the number of living/dead cells using very different structural or functional hallmarks of cell viability as readout. They commonly make use of colorimetric or fluorogenic labels to assess the viability status; label-free assays based on changes in cell morphology have been described as well.

3.1. Label-Based Assays to Assess Cytotoxicity

Table 1 summarizes the most widely used label-based assays to quantify the fraction of live cells in a given population at a fixed endpoint of the exposure. The label-based endpoint assays compared in **Table 1** are categorized in three classes: (*a*) assays addressing membrane integrity, (*b*) assays addressing the metabolic activity of the cells, and (*c*) assays indicating apoptosis.

Membrane integrity is an unconditional prerequisite for cell viability. Therefore all assays that monitor the permeability of the plasma membrane report on cytotoxicity. They measure the uptake of membrane-impermeable probes from the extracellular fluid into the cytoplasm or the efflux of intracellular molecules into the supernatant. Typical probes that are applied to the extracellular fluid are trypan blue and naphthalene black. Both reagents stain cells with impaired membrane integrity such that dead cells are identified easily by bright-field microscopy (12). More sensitive microscopic identification of dead cells can be achieved using fluorescent probes such as propidium iodide or ethidium homodimer. If membrane integrity is compromised, these membrane-impermeable dyes have direct access to the nucleus, intercalate into the DNA, and mark dead cells with bright nuclear fluorescence (12).

In contrast, the lactate dehydrogenase (LDH) assay measures the activity of the cytoplasmic enzyme in the supernatant by coupling the enzymatic reaction to the formation of a colored or fluorescent probe. Monitoring the leakage of a cytosolic enzyme as an indicator for a loss of membrane integrity is a significantly more sensitive approach than following the influx of a stoichiometric colorimetric probe, given the inherent amplification in enzyme detection (12). Similar assays such as the LDH assay have been developed that measure the efflux of other enzymes [glucose-6-phosphate dehydrogenase (G6P-DH) or glycerinaldehyde-3-phosphate dehydrogenase (GAP-DH)] by their concentration in the supernatant (12). However, probing of LDH in the supernatant, as with G6P-DH and GAP-DH, renders this assay inappropriate for all CMA formats with one common bathing fluid. This conclusion applies to all assays that rely on analysis of the supernatant, as they do not allow for a spatially encoded readout necessary to assign a given cytotoxicity measure to an individual μ-spot on the array.

The so-called MTT assay—with MTS and WST being closely related—is the most widely applied assay to measure acute toxicity by probing the metabolic activity of the cells (12). MTT, MTS and WST are acronyms for the indicator dyes used in these assays. It measures the cells' pool of the redox coenzymes NADH and $FADH_2$ as an indicator for the cells' mitochondrial performance. The assay relies on the reduction of colorless tetrazolium salts to deep red formazan, which can be easily quantified. Variants of this assay such as Alamar Blue™ or Presto Blue™ follow the same strategy but use fluorogenic indicators for the redox reactions. They all suffer from the same limitation as described for the enzyme leakage assays (e.g., LDH) and cannot be applied to CMAs, as they quantify the viability indicator in the supernatant.

Another class of metabolic assays used to assess cell viability is, however, readily applicable to CMAs. They are based on membrane-permeable but nonfluorescent reagents such as calcein

Table 1 Established label-based assays to distinguish live and dead cells

Indicator	Reagent(s)	Mechanism	Detection	CMA suitable
Membrane integrity				
Dye exclusion assays	Naphthalene black	Cytoplasm of dead cells with permeable membranes is stained by the membrane-impermeable dye	Bright-field microscopy of blue/black stained dead cells	Y
	Trypan blue			Y
	Propidium iodide	Nuclei of dead cells with permeable membranes are stained by the membrane-impermeable dye given dye intercalation in DNA	Red fluorescence from nuclei of dead cells	Y
	Ethidium homodimer-1			Y
Enzyme leakage assay, e.g., LDH	Lactate, NAD^+, diaphorase, resazurin	Cytosolic housekeeping enzyme LDH is released from cells with damaged membranes and catalyzes oxidation of lactate to pyruvate in extracellular buffer producing NADH[a]	Fluorescence or absorbance in extracellular supernatant	N
Metabolic activity				
Reducing coenzymes (NADH, NADPH, $FADH_2$, $FMNH_2$)	Tetrazolium salts MTT (is) MTS (s) WTT (s)	Reduction of yellow water-soluble tetrazolium salts by reducing coenzymes to (is) or (s) formazan dyes	Absorbance of solubilized formazan	N
	Resazurin (e.g., alamarBlue®, PrestoBlue®)	Reduction of nonfluorescent membrane-permeable resazurin (blue) by reducing coenzymes to membrane-permeable red-fluorescent resorufin	Fluorescence or absorbance in extracellular medium	N
Enzyme activity (e.g., intracellular esterase activity)	Fluorescein diacetate	Intracellular hydrolysis of membrane-permeable precursor (fluorescein diacetate, calcein AM) by cellular esterases to the membrane-impermeable fluorophore (fluorescein, calcein) trapped inside the cell	Green cytoplasmic fluorescence of stained viable cells	Y
	Calcein AM			Y
ATP level	Luciferase, luciferin	ATP-dependent conversion of luciferin to luminescent oxyluciferin	Luminescence in cell or cell hydrolysate	Y
pH gradients (ATP level)	Neutral red	Neutral red accumulates in lysosomes due to ATP-driven pH gradients in living cells	Absorbance of solubilized stain	N
Apoptosis				
Caspase activity	Peptide with specific recognition sequence for caspase embraced by a FRET pair	Caspase-3 (or -9) from cell lysates cleaves the test peptide, providing strong fluorescence that is quenched in intact peptides	Microplate reader for fluorescence	N

(*Continued*)

Table 1 (*Continued*)

Indicator	Reagent(s)	Mechanism	Detection	CMA suitable
DNA fragmentation	DAPI	Dyes bind to DNA and indicate nuclear morphology and fragmentation	Fluorescence microscopy	Y
	Hoechst 33258			
	Hoechst 33342			
	—	Chromosomal DNA studied for fragmentation by agarose gel electrophoresis	Gel staining	N
Phosphatidylserine	Annexin-A5	Fluorescently labeled Annexin-A5 binds to phosphatidylserine in extracellular leaflet of plasma membrane	Fluorescence microscopy for adherent cells/cytometry for suspended cells	Y
TUNEL	t-Deoxynucleotidyl-transferase + fluorescence-labeled dUTP	Fluorescence-labeled dUTP will be transferred to free DNA ends resulting from DNA strand breaks	Fluorescence microscopy	Y

Abbreviations: AM, acetoxy-methylester, ATP, adenosine triphosphate; CMA, cell microarray; DAPI, 4,6-diamidino-2-phenylindole; FAD, flavin-adenine-dinucleotide; FMN, flavin-adenine-mononucleotide; FRET, Förster resonance energy transfer; (is), water insoluble; LDH, lactate dehydrogenase; MTS, MTT, WTT, abbreviations for metabolic assays; NAD, nicotinamide-adenine-dinucleotide; NADP, nicotinamide-adenine-dinucleotide-phosphate; (s), water soluble; TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling.
[a]NADH is used to reduce resazurin to fluorescent resorufin catalyzed by diaphorase.

AM, which are hydrolyzed by esterases inside metabolically active cells. The resulting calcein is green fluorescent but no longer membrane permeable such that it accumulates in the cytoplasm. Dead cells are unable to catalyze metabolic conversion of calcein AM and thereby do not obtain green cytoplasmic fluorescence as an indicator for cell viability (13). A variant of this assay uses fluorescein diacetate instead of calcein AM (12), but it follows the same principles.

Other metabolic assays read the ATP levels inside the cells as an indicator for viability. This occurs either directly or indirectly. Measuring the chemoluminescence associated with the ATP-dependent conversion of luciferin to oxyluciferin provides a direct readout. The neutral red (NR) uptake is a more indirect assay but it also addresses the intracellular ATP supplies as a measure for metabolic status. NR accumulates in the lysosomes as long as the pH inside the lysosomes is lower than in the rest of the cell due to the activities of ATP-driven proton pumps. Thus, the more ATP is available, the more pronounced is the pH gradient across the lysosomal membrane and the stronger is the NR uptake. Thus, the amount of NR is directly correlated to ATP levels inside the cells (14). Both viability assays reading intracellular ATP levels are applicable to CMA formats. However, the chemiluminescence of the luciferase reaction or the NR staining has to be recorded microscopically.

The assays described above measure the integrity of the plasma membrane or rather drastic changes in energy metabolism. The rationale for the membrane integrity assays is the general observation that necrotic cells swell and their membranes eventually rupture, leading to cell lysis and a loss of homeostasis. In contrast to necrosis, which is a more accidental but still regulated cell death (15) caused by toxins or unfavorable external conditions (temperature, pH, ionic strength), apoptosis is the genetically encoded, strictly regulated cell suicide during which the cells digest themselves (16–18). The cell bodies shrink, the membrane shows characteristic blebs but remains

intact, and the nucleus and other intracellular components are degraded and packed into vesicles (19, 20). In an intact organism, these vesicles can be safely removed without intracellular components getting into the extracellular space. In vitro apoptotic cells will eventually also suffer from a permeabilization of their plasma membrane at very late stages. Although toxicologists have traditionally associated cell death with necrosis, emerging evidence suggests that different types of environmental contaminants exert their toxicity, at least in part, by triggering apoptosis (17). Besides other features necrotic and apoptotic cells can be distinguished by their ultrastructural morphology (swelling versus shrinking), the differing integrities of their plasma membranes, the appearances of their nuclei and the activation of apoptosis-specific proteases, the so-called caspases that pursue self-degradation in cells. Accordingly, several assays have been established to screen a given cell population for its degree of apoptosis (21). Some of these assays are integral in nature; i.e., the assays measure the activity of the intracellular caspases or the degree of DNA fragmentation integrated over the entire cell population. These are not suitable for application in CMAs for reasons discussed above. Other assays label individual apoptotic cells in a cell population, so that the number of positive cells can be quantified by microscopy. One of the most widespread assays of this kind is the so-called Annexin-A5 assay, which is based on the specific binding of fluorescence-labeled Annexin-A5 to the plasma membrane of apoptotic cells (22). This membrane binding occurs as apoptotic cells flip phosphatidylserine (PS) from the inner leaflet of the plasma membrane, where it is exclusively localized in healthy cells, to the extracellular outer leaflet of the membrane. After this very early indicator of apoptosis becomes visible from the extracellular side, Annexin-A5 can bind to PS and label this particular cell. The so-called TUNEL assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) labels apoptotic cells with respect to the fragmented DNA (23). Enzymatic fragmentation during apoptosis leaves the DNA with free 3′-OH ends that are targets of terminal deoxynucleotidyl transferase that adds dUTP to the free 3′-OH. When fluorescence-, biotin-, or Br-labeled dUTP is provided during the assay, the free DNA ends will be marked in situ such that DNA fragmentation is visible by microscopic inspection either directly, as in the case of fluorescence-labeled dUTP, or indirectly, as after streptavidin or antibody tagging.

In contrast to apoptosis, no distinct surface or biochemical markers of necrotic cell death have been identified yet besides the observation of cell death in combination with the absence of apoptotic parameters, as mentioned above. Vanden Berghe et al. (21) provide a concise review about bioanalytical assays to distinguish between apoptosis and necrosis.

Even though these indirect colorimetric assays do not require cell removal from the growth surface and can be applied to cells adherently grown on a CMA, they are all endpoint assays as the cell population has to be sacrificed by the addition of the experimental reagents. Time-resolved analysis of toxicity based on these assays is only possible by running several assays sequentially at different time points. The only experimental option to study the time-dependent dynamics of the toxicological response is to use cells that carry an endogenous reporter system (see Section 2.3) that is not harmful to the cells or to use label-free readout approaches.

3.2. Label-Free Approaches to Monitor Cell-Based Assays

The most obvious label-free approach to study cells during exposure to drugs or toxins is light microscopy, which is noninvasive and reports on changes in cell shape or morphology and optical thickness (24). As cells do not absorb visible light to a measurable degree, it requires phase contrast, digital interference contrast, or digital holography to visualize the cells on appropriate supports without using any labels. All three microscopic techniques are suitable to perform a phenotypic screening of cells cultured on a CMA, and successful examples have been described (25, 26). In all

cases, cell morphology is the integral, holistic bioanalytical indicator for the onset of cytotoxicty and cell dysfunction without any molecular specificity (27). This unspecific integral information is often considered the weakness of holistic approaches such as the ones mentioned above, but these techniques do not rely on any a priori knowledge about the molecules involved in the cellular response to a given stimulus and are, thus, entirely unbiased. One approach has attracted considerable attention recently: digital holographic microscopy (DHM) (28–30). DHM provides label-free 3D images of transparent objects such as living cells and is thus perfectly suited for phenotypic screening based on cell morphology. DHM has been applied just recently in 96-well formats (29), a development that paves the way for high-throughput applications such as analysis of CMAs.

Although it is not usually considered a classical light microscopy technique, surface plasmon resonance imaging (SPRi) has also demonstrated its potential to monitor CBAs with lateral resolution (31). Published examples consist of successful monitoring of the cell response to stimulation of cell surface receptors (32, 33) or encounters with allergens (34). The imaging variant of classical SPR is considered another prospect for imaging the cell response on CMAs without using labels. SPR essentially reads changes in the refractive index (RI) close to the growth surface. Changes in RI during CBAs are generally attributed to a dynamic mass redistribution most often associated with changes in cell morphology. Thus, the information content in SPRi is similar to that in light microscopy–based readouts. Conventional, nonimaging SPR (35, 36) or devices based on resonant waveguide grating (37) have proven their outstanding performance in monitoring CBAs (38, 39). Waveguide devices have been used for 384-well formats and contributed significantly to pharmacology as a transducer for cell phenotyping. No obvious scientific or technical reason prohibits the development of these devices to become monitoring technologies for CMAs. However, microarray substrates have to be compliant with the optical requirements of the technique. Moreover, the cells have to be cultured in close proximity to the surface, as the method is blind for any change in RI that originates farther away from the surface than 200 nm (evanescent field technique). Analysis of 3D cell aggregates will thus be difficult if not impossible.

Another label-free approach for monitoring CBAs relies on electrochemical impedance measurements. The technique is referred to as electric cell-substrate impedance sensing (ECIS) (40). In ECIS, cells are grown on planar gold-film electrodes that are deposited on the bottom of cell culture dishes. The electrical impedance of these electrodes changes when adherent mammalian cells attach and spread on the electrode surface, as the current is forced to flow around the insulating cell bodies. Bypassing the cell bodies on extracellular ionic pathways gives rise to impedance in the narrow cleft underneath the cells and in the intercellular cleft between adjacent cells. Thus, the recorded impedance signal is highly sensitive to changes in cell morphology, as any change in cell shape alters the extracellular ionic current pathways (41). It has been estimated by model calculations that cell shape changes far below the resolution of an optical microscope are measurable and detectable using the ECIS technique. ECIS has been successfully applied to various CBAs from cell adhesion to protein layers (42), cell proliferation, cell migration (43), and cell responses to toxins (44–46). ECIS was successfully applied as a transducer to monitor the morphological changes of sensor cells during chemically induced apoptosis; it indicated the onset of apoptosis more sensitively than the classical biochemical assays described above (47). State of the art with respect to parallelization and throughput are commercial devices that allow using ECIS in 96-well or even 384-well formats. As each electrode used in the assay needs to be contacted individually, it is hard to envision a further increase in parallelization. Moreover, further upscaling in throughput requires further downscaling of electrode sizes. Electrodes smaller than 100 μm in diameter are, however, more demanding with respect to the impedance analyzers as the absolute impedance values increase with decreasing electrode radius and parasitic contributions become harder to suppress or control. Although impedance-based cellular assays can already

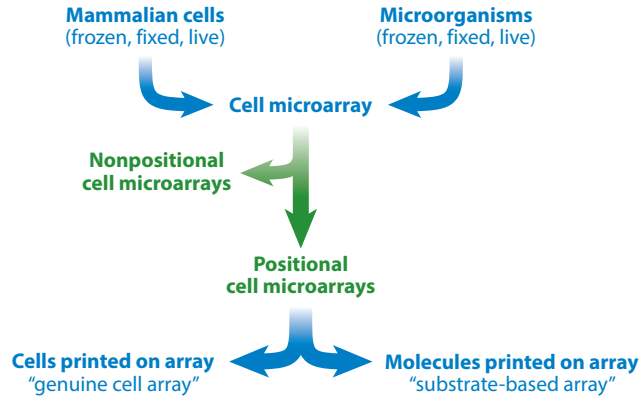

Figure 2

Technical classification scheme for cell microarrays.

be performed with high-throughput in 384-well MTP formats, application of the impedance technology to CMAs with a significantly higher spot density will remain a challenge in the future.

4. CELL MICROARRAY FORMATS

CMAs have been described for mammalian (e.g., human) cells (48, 49) and for microorganisms (50). CMAs harboring microorganisms are often referred to as microbial microarrays. This review discusses toxicity assessment and focuses entirely on mammalian CMAs; the interested reader is referred to Belkin and coworkers' (50, 51) comprehensive surveys about microbial microarrays. From a technical viewpoint, CMAs are categorized into two major classes (48), nonpositional and positional CMAs (**Figure 2**).

4.1. Nonpositional Microarrays

Figure 3 illustrates the principle of performing CBAs with nonpositional CMAs. The general idea of this approach is to mix a selection of different cell types in one culture dish, to expose this cell mixture to a particular experimental stimulus (e.g., a given toxin in a predefined concentration), then to measure and identify the individual responses of the different cell types to this common challenge simultaneously. Because the cells are not immobilized and addressable on a solid substrate but float around in the experimental solution, they need to be individually labeled before cell mixing for later identification.

Prelabeling of the individual cell types can be performed with the help of fluorescent antibodies that recognize cell-type-specific cell surface antigens and mark the cell type by color (48). Alternatively, cell-type-specific labeling can be provided by internalization of quantum dots (QDs) of different sizes with different emission properties before the cells get mixed into the common reaction volume. Mattheakis et al. (52) used QDs that had been surface functionalized by virus-derived cell-penetrating peptides that enhanced the uptake of the QDs into the cells under study. Because QDs are particularly photostable, they provide long-term luminescence labeling of each cell population. By using well-defined individual mixtures of differently emitting QDs for each cell population, the authors were able to provide more than 100 different individual emission codes to label a corresponding number of cell types. Thus, more than 100 different cell types can be studied in parallel under exactly the same conditions in one culture dish. The approach was successfully

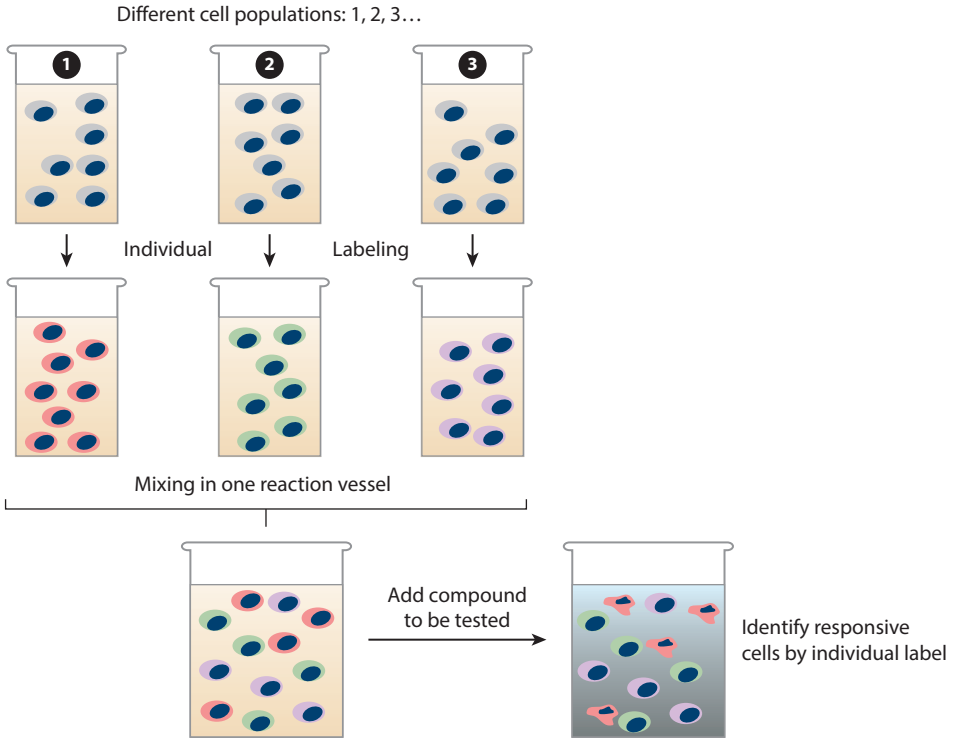

Figure 3

General scheme for the performance of nonpositional cell microarrays.

applied to different kinds of CBAs addressing the activation of cell surface receptors, expression of reporter genes, or cell-cell interactions (52).

A different approach for nonpositional encoding of unique cell types is the CellCard System (53–55). Here the cells are grown on small chips or "cards" that provide room for the adhesion of a small number of cells (<100). Each cell type is grown as pure culture on its own cards. Each card is color-coded at the side, so that the cells are identifiable under the microscope even after the cards loaded with different cell types have been mixed. The readout is performed by automated microscopy and image analysis, which may be the bottleneck for applying this technology in large screening campaigns. A published example (53) describes the use of ten different cell types, encoded by growth on individual cell cards, in every single well of a 96-well MTP. This way the 96-well plate allows analyzing 960 different combinations of cell types and incubation conditions. According to the authors, the technology is applicable to almost every CBA that can be monitored and quantified by microscopy. One concern is that the various cell types are bathed in the same reaction buffer, which allows for chemical communication between the different cell types that would not be possible in individual wells of a regular MTP. This may become a serious problem in cytotoxicity assays when more susceptible cells die before the more resistant ones and release their intracellular enzymes into the incubation buffer. Proteases, nucleases, peptidases—any of the hydrolases—may affect the cells in the shared incubation buffer, potentially inducing a cell response that is not based on the chemical under study. However, this problem is common to all CMAs that operate in one common volume. An interesting alternative to the original idea of using cell cards with color codes imprinted on their sides is the use of cards with different shapes (56).

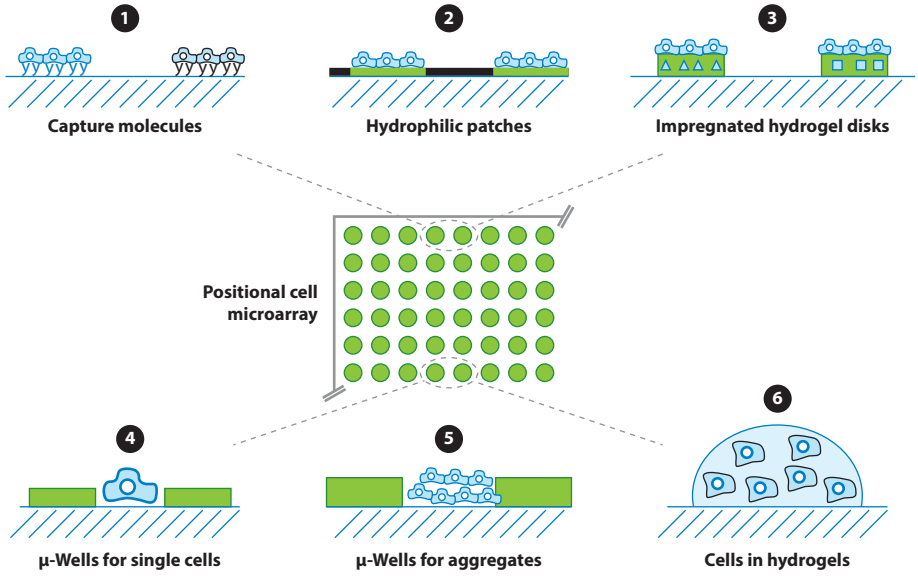

Figure 4

Various positional cell microarrays. In types ❶, ❷, and ❸, adhesive (or repulsive) molecules are prearrayed on the substrate; in types ❹, ❺, and ❻, cells are arrayed on the substrate.

Here, the different cell types are encoded by the shape of the chip on which they are growing. The number of possible codes is enormous given various irregular shapes can be realized with the use of modern microfabrication techniques. Both approaches, cards encoded by color strips and those encoded by shape, are superior to the above mentioned QD approach, as they do not interfere with any fluorescence readout that might be necessary to quantify and indicate the cell response.

4.2. Positional Cell Microarrays

In positional CMAs, reagents or cells are deposited in small spots on a solid support such that the individual reaction between reagent and cell is addressable by the *xy*-coordinates of the individual μ-spot (57). When different reagent molecules are arrayed on the solid support before the cells are allowed to settle, the resulting microarrays are referred to as substrate based; when different cells are arrayed before the reagent solution is added, the resulting microarrays are referred to as genuine CMAs (48). **Figure 4** summarizes the various types of positional CMAs that we discuss briefly below.

4.2.1. Arrays of capture molecules. Specific capture molecules (e.g., antibodies, ligands to cell surface receptors, extracellular matrix proteins) are deposited in a highly ordered array of μ-spots on a solid support with standard spotting techniques. The identity of the capture biomolecules is encoded by their individual position on the microarray, i.e., their individual *xy*-coordinates. The space in between the spots is kept nonadhesive to avoid nonspecific attachment of cells to the array. When a suspension of cells is flooded across this array surface, all cells presenting a corresponding binding partner for the prearrayed molecules on their plasma membrane bind to the surface-immobilized counterpart and are captured from the suspension. Examples include arrays of (*a*) antibodies targeting specific cell surface epitopes (58), (*b*) glycans specific for cell

surface lectins (59), (*c*) lectins specific for cell surface glycans (60), and (*d*) MHC (major histocompatibility complex)-peptides recognizing antigen-specific T-cells in whole blood or mixed T-cell populations (61). Presence of a molecular cell surface marker is indicated by binding of cells to the corresponding μ-spot and is analyzed by microscopic counting or fluorescence labeling. Analysis of the entire CMA provides a detailed profile of the individuals within the cell population under study. Depending on the identity and number of recognition molecules, such an array can be used for providing detailed characterization of a cell suspension with respect to the population's cell surface markers (62).

These approaches can be used for not only population profiling, but also for preparing arrays with a maximum number of different cells that can then be screened for their functional response to a chemical that is simultaneously applied to all cells on the CMA via the bathing fluid. So in contrast to screening chemical libraries on one cell type, here a library of cells from different organs or species is screened for a functional response to a given chemical (62).

A nonspecific method of anchoring cells to a μ-spot uses what is called a biocompatible anchor of membrane (BAM). In this approach a cheap and abundant protein like serum albumin is microarrayed on a solid support. The BAM consists of a fatty acid such as oleic acid coupled via a polyethylene-glycol (PEG) spacer to an amino reactive *N*-hydroxy-succinimdyl group that covalently binds to the predeposited protein with the fatty acid facing toward the supernatant. Upon cell encounter, the oleic acid will integrate into the plasma membrane and thereby capture the cells nonspecifically to the array (63). This approach does not provide any significant discrimination between different cell types.

4.2.2. Arrays of hydrophilic patches. Instead of using spots of highly specific capture molecules, it is also possible to establish an array of hydrophilic spots on a hydrophobic background (48). When adhesive proteins are flooded across such a patterned surface, they will preferentially adsorb in native form on the hydrophilic patches providing attachment sites for suspended cells. Protein adsorption on hydrophobic surfaces often leads to protein unfolding, which renders a surface nonadhesive. The advantage of these arrays in comparison to confluent cell monolayers is the ability to make individual cell colonies addressable by the *xy*-coordinates of their μ-spot. Similarly, predeposition of adhesive proteins in array format leads to the preferential adhesion of cells to these sites. When the periphery is made nonadhesive, the cells do not outgrow the size of the protein spot. Note that these kinds of arraying techniques have been used to screen cell populations for their preferential adhesion to different combinations of adhesive proteins or biomaterials used for implants and scaffold materials (64). This CMA-based screening for adhesion preferences has received considerable attention in stem cell research and tissue engineering as it helps to find the proper microenvironment for cell differentiation.

4.2.3. Arrays of impregnated hydrogel disks. One of the technical challenges for the routine use of CMAs to assess cytotoxicity is the delivery of individual compounds of a chemical library to the individual μ-spots of the CAM without cross-contaminating the neighboring sites. Above, we described how arrays of different cells can be generated and exposed to one chemical in a predefined concentration as long as this chemical is delivered via the bathing fluid; however, screening a chemical library for toxicity on one cell type requires the particularly local and exclusive administration of individual compounds to one μ-spot.

One approach is based on arrays of small hydrogel disks ($d = 200$ μm) made from biocompatible poly-D,L,-lactide-glycolide copolymers that are individually impregnated with the chemical to be studied (65). The cross-linked hydrogels allow out-diffusion of the compounds when cells from a homogeneous suspension were allowed to adhere to the individual adhesive hydrogel spots.

Reading the cell response at a given *xy*-coordinate of the array provided the response of the cells to this compound predeposited at this position of the array. The impregnated hydrogel spots may not only contain the substance to be tested but also assay reagents that are necessary for analysis of the cell response, as described below.

Almost the inverse strategy has been described by Gopalakrishnan et al. (66) who arrayed a library of more than 8,500 chemicals on a polystyrene support by robotic printing. Suspended HEK293 cells were embedded in an agarose gel with the membrane-permeable indicator FLUO-4, which is commonly used to measure the intracellular Ca^{2+} concentration. This agarose gel doped with cells, and the assay reagents were then blotted onto the polystyrene support so that the compounds could diffuse into the gel to reach the cells. The study was not meant to screen for cytotoxicity but for the activation of cell surface receptors that induce an increase in intracellular Ca^{2+} upon ligand binding. This example of screening a compound library for receptor activation is, however, transferable to screening a compound library for toxic compounds. All it takes is a change of the readout assay. Instead of receptor activation (intracellular Ca^{2+} concentration), the cells in the agarose gel have to be screened for cell death, for instance, by calcein AM/ethidium homodimer costaining. However, there is concern about cross-contamination, as the compounds undergoing testing are free to diffuse in all directions within the agarose gel.

4.2.4. Arrays of μ-wells for single cells, multiple cells, or cell aggregates. Living cells can be arrayed directly on a solid support from a monodisperse cell suspension with the help of a piezo-nozzle, hollow pins, or inkjet devices (67). As a prerequisite for these approaches, the array surface has to promote adhesion so that the cells can anchor at the site of their deposition. Inkjet printing is, however, stressful for suspended cells as it requires applying shear, suction, or pressure and requires gentle operations and great care. Wilson & Boland (67) managed to print and deposit even primary cultured cells that survived after inkjet printing for more than 72 h. Later Xu et al. (68) showed for different cell types that only a small fraction (<10%) of the individual cells got ruptured and killed by the spotting procedure. When printed cells are used for toxicology assessment, it is mandatory to ensure that the printing process does not provide any significant trauma to the cells, as this could create false-positive readouts of cytotoxicity.

Cell positioning is also achieved by dielectrophoresis (DEP) (69). The dielectric cell bodies respond to inhomogeneous electric fields by moving along the field gradient as long as they are suspended. Gray et al. (69) have positioned individual cells from suspension and allowed the cells to adhere to the adhesive array substrate when their individual position was reached. Albrecht et al. (70) used the same principles to position cells in 3D within a non-cross-linked hydrogel. Once all cells had reached their final position, they got captured by photo-cross-linking within the hydrogel. The only disadvantage of the DEP-based cell positioning for high-throughput applications is the necessity for particularly complex electrode layouts on the array surface to provide the electric fields necessary for the controlled movement of suspended cells. Contactless DEP devices have been described (71, 72) and may offer new strategies for the future.

A compromise between spotting of individual droplets of suspended live cells or suspended live cell aggregates and predeposition of capturing molecules that capture cells from suspension might be the development of μ-well arrays. These μ-wells are typically 50 to 200 μm in diameter. The dimensions of the wells generated in array format are tailored for single cells or 3D cell aggregates such as tumor spheroids (73). They are often produced from photosensitive PEG derivatives (74), chitosan (75), or hyaluronic acid (76), which are cross-linked upon illumination. Alternatively, these arrays are prepared by soft lithography/microfabrication using other cell-repellent or biocompatible materials such as polydimethylsiloxane (PDMS). The procedures provide arrays with several thousands of these μ-wells in precisely defined array formats. The μ-wells allow a softer

seeding of cells or cell aggregates, reducing the fear of cell trauma prior to the assay. Cells (one or more) or cell aggregates are delivered into the individual μ-wells with computer-controlled liquid-handling approaches.

Exposure to individual members of a compound library for cells cultured in an array of μ-wells has been achieved by printing the molecules of interest on the tips of μ-posts that are scaled to match the dimensions of the μ-wells. The compounds are added to the cell-loaded μ-wells by sandwiching the two parts together such that the posts seal the individual wells and the cellular response starts to emerge in a reaction volume as low as 20 nL. Cell viability is quantified by the fluorescence-based calcein AM/ethidium homodimer assay (see **Table 1**). The cells are shown to be responsive under these conditions for at least 24 h (77).

4.2.5. Arrays of hydrogel droplets with encapsulated cells. Instead of using μ-wells to immobilize suspended cells, some strategies prefer their encapsulation in cytocompatible hydrogels such as alginate, collagen, agarose, or gelatin (78, 79). It is important from an experimental perspective that the cells are suspended in the hydrogel with gelation only starting once the droplet has been deposited on the array surface. Lee et al. (80) reported a special protocol that ensures immediate gelation on the array surface but not any sooner. Here, the cells were suspended in cytocompatible alginate and the alginate droplets were then deposited in very small volumes on the base support of the CMA. The authors precoated the surface with poly-L-lysine doped with Ba^{2+} ions. The polycation poly-L-lysine served as a buffer to immobilize the divalent Ba^{2+} ions and electrostatically attract the negatively charged alginate. Once the cell containing alginate droplets (20 nL) made contact with the substrate, the Ba^{2+} ions induced immediate gelation so that the droplet was immobilized with the cells contained inside. The alginate gel was permeable enough for the diffusion of low-molecular-weight compounds tested but kept the cells and the liquid in place (80).

5. CELL MICROARRAYS IN TOXICOLOGY ASSESSMENT: TWO CASE STUDIES

Two published studies have been selected to demonstrate the potential of CMAs for toxicology assessment in more detail. A CMA approach with outstanding information depth with respect to toxicology is the metabolizing enzyme toxicology assay chip (MetaChip) concept (80, 81). It takes into account that some drugs or chemicals may not be toxic in their original form but get metabolically transformed into a toxic species—most often in the liver by the cytochrome P450 (CYP) family of liver enzymes. The authors tried to account for this phenomenon by spotting one member of the liver-specific CYP enzyme superfamily, namely CYP3A4 and later CYP2B6 and CYP1A2, embedded in a gel matrix on a solid support. Gel spots without any CYP enzymes served as controls for the toxicity of the nonmetabolized compounds. The compounds of interest were then deposited in predefined concentrations on top of these gel droplets and were allowed to diffuse into the gel where they are potentially metabolized by CYP. After a predefined incubation time, the array of chemicals and their potential metabolites were stamped onto a confluent monolayer of MCF-7 cells (human breast cancer cell line) that served as the in vitro toxicology text system (81). After an incubation time of 6 h, the cells were tested for their viability using the calcein AM/ethidium homodimer vital stain (see **Table 1**). These studies revealed the cytotoxicity of the pure, nonmetabolized compounds together with the impact of their potential metabolites produced by the presence of CYP enzymes. This readout provides more realistic data on the impact of suspicious compounds on cell physiology, as it includes at least in part their metabolic conversion.

In a subsequent development step, the MetaChip that holds the compounds in combination with different CYP isoforms was combined with a so-called data analysis toxicology assay chip (DataChip) that replaced the continuous monolayer of MCF-7 cells. This is a CMA with 1,080 individual μ-spots, each of which contains cells—either MCF-7 or cells of the liver cell line Hep3B—dispersed in 20 nL of an alginate hydrogel. Thus, the DataChip is a 3D CMA and no longer a 2D monolayer. (Preparation of the cell array is discussed in Section 4.) The chip with the 3D cell cultures was used in conjunction with a MetaChip as described above but with the geometry and spot density matching those of the DataChip (80). Sandwiching these two complimentary arrays brings the cells in contact with the compounds, and after a given incubation time the cell response to the toxin encounter is quantified again by calcein AM/ethidium homodimer staining. **Figure 5** illustrates the MetaChip/DataChip concept and the experimental procedure to record cytotoxicity profiles of the test compounds. Experiments yielded dose-response relationships for nine compounds and their metabolites from four different CYP conversions at a quality sufficient to determine the individual IC_{50} values for each incubation (**Figure 6**). Results were shown to be in good agreement with assays performed in regular 96-well MTPs and also with in vivo data (82). The latter finding is particularly noteworthy given it validates the CMA approach for

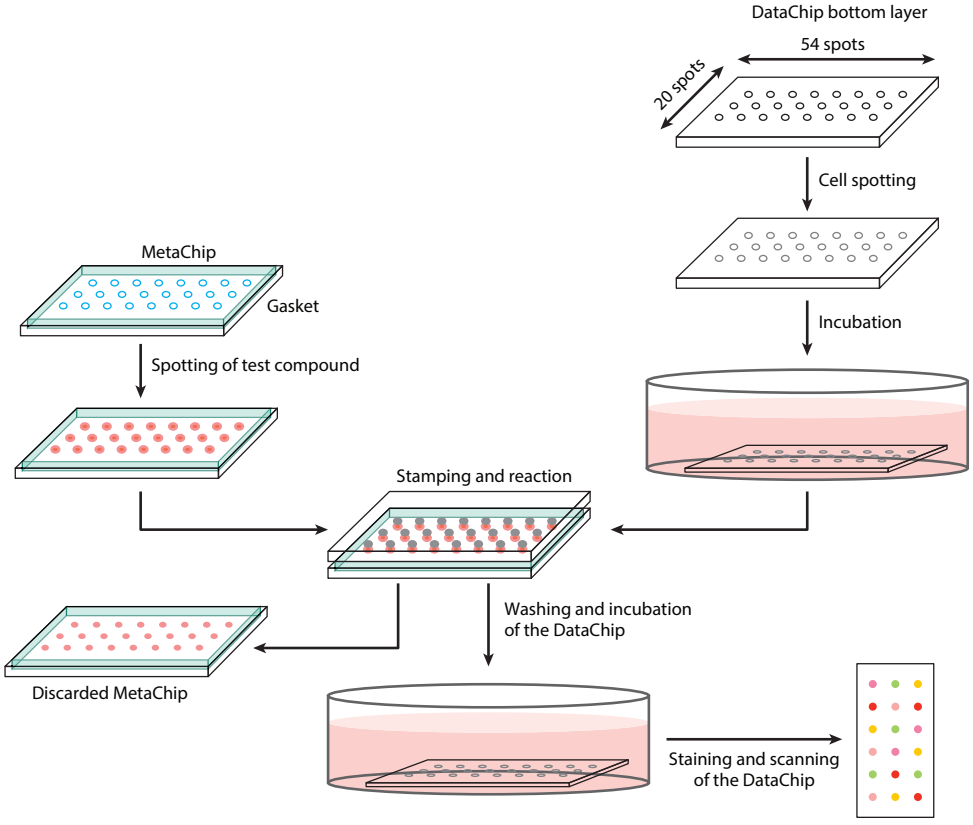

Figure 5

Schematic of the MetaChip/DataChip platform for cytotoxicity testing of chemicals and several of its liver metabolites. Figure reprinted with permission from Reference 80.

toxicology assessment, not only for chemicals as they are absorbed by the body but also for their liver metabolites.

The second case study involves a low-tech, bench-top approach for screening chemical libraries for compounds that induce apoptosis or necrosis—the two mechanisms of cell death (83). The technology is also based on the use of two separate arrays, one for arraying cells and the other for arraying the chemical library. Colonies of MCF-7 cells were deposited in an array of 2,100 μ-wells microfabricated from PDMS on a standard microscope slide. Each well has a diameter of 400 μm and a depth of 300 μm. The chemical compounds were impregnated into 2,100 individual droplets of photo-cross-linked PEG diacrylate. The hydrogel droplets matched exactly the cell-loaded μ-wells and were able to seal the wells upon sandwiching the two arrays together, which is the starting point of the cell-based toxicity assay. After 12 h of incubation, the cells were tested for apoptosis and/or necrosis using the Annexin-A5 assay (see **Table 1**), indicating apoptosis and a variant of the ethidium homodimer assay reporting on membrane integrity and indicating necrosis. Analysis of the assay has been performed using an automated array scanner, and from the fluorescence intensities measured the authors could conclude on the toxicity of the compounds under study and on the mechanism of cell death that is induced by the various chemicals. A set of five chemicals has been examined in different concentrations. The experiment provided a relative ranking of the chemicals to induce apoptosis or necrosis (83).

6. CONCLUDING REMARKS AND CRITICAL ANALYSIS

Application of CMAs for in vitro toxicology or other functional assays addressing cell physiology is still in its infancy and requires more technical progress to become a routine technology. Only a few examples (see Section 5) have been described that are beyond the proof-of-concept level. Cell microarrays, however, have been widely applied for cell profiling, i.e., the structural analysis of cell surface molecules such as glycans, lectins, T-cell receptors, or adhesion receptors (62). With the enormous spot density realized on molecular microarrays, it is possible to screen for thousands of cell surface markers in a single experiment. In this respect, cell microarrays are much more powerful than conventional cytometry approaches, which are heavily dependent on and limited by the availability of fluorescent labels with sufficiently separated spectroscopic characteristics. But it is noteworthy that immobilization of capture molecules, such as antibodies, on a planar surface may change their binding affinity and conformational flexibility (58). In this respect the microarray approach may be inferior to cytometry as the latter relies on molecular recognition in the bulk phase.

Another highly successful application of CMAs is reverse transfection arrays (84). Here, plasmid DNA encoding a protein of interest is spotted on a microarray together with a transfection reagent and an adhesive protein such as poly-L-lysine or gelatin to enable cell adhesion. When initially suspended cells attach to the adhesive μ-spots on the array, they take up the plasmid DNA deposited on their growth support with the help of the codeposited transfection reagent. Subsequently, the cells start expressing the new genetic information and produce the encoded protein. With the help of these reverse transcription microarrays, thousands of different genes can be expressed in parallel to study the associated changes in cell physiology (gain of function) (85). In a technically similar but functionally inverse approach, the cells are transfected with predeposited siRNAs that are capable of knocking down the expression of one particular protein (loss of function) (86). These genomic microarrays require a functional readout revealing the changes in cell physiology (e.g., cell proliferation, differentiation, or apoptosis) that are associated with the expression or repression of one specific gene.

Compared to these established routine CMA applications, the use of CMAs in toxicity assessment is only in its infancy; however, expectations and promises are high. It is undisputed and

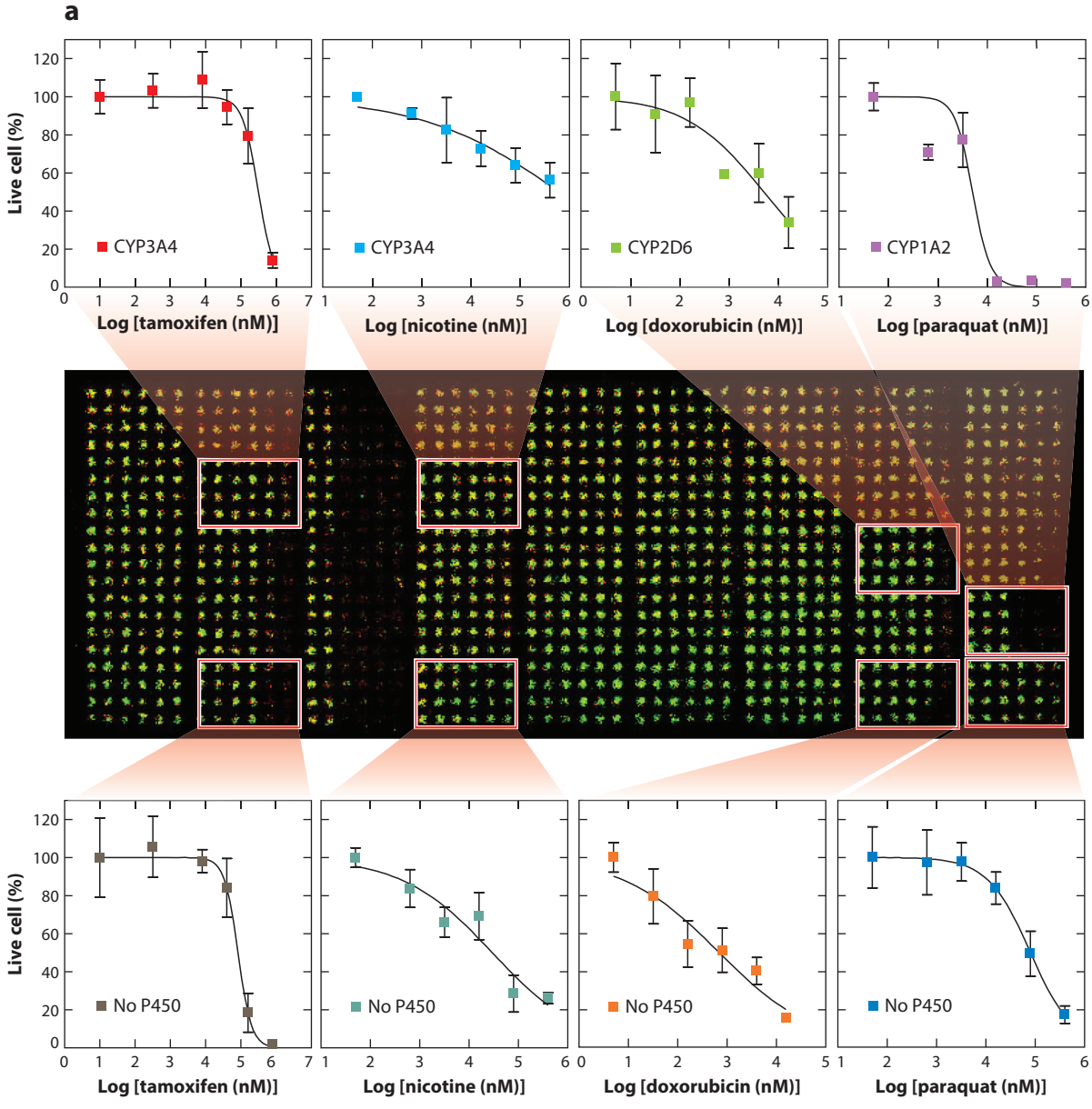

Figure 6

Analysis of the DataChip to establish cytotoxicity profiles of nine different chemicals using the human liver cell line Hep3B. (*a*) Analysis of a submatrix of spots on the DataChip to produce dose-response curves of each of the nine chemicals. (*b*) Cytotoxicity profile (IC_{50}) for nine chemicals with or without metabolization by the indicated CYP enzymes or their mixture. Figure reprinted with permission from Reference 80.

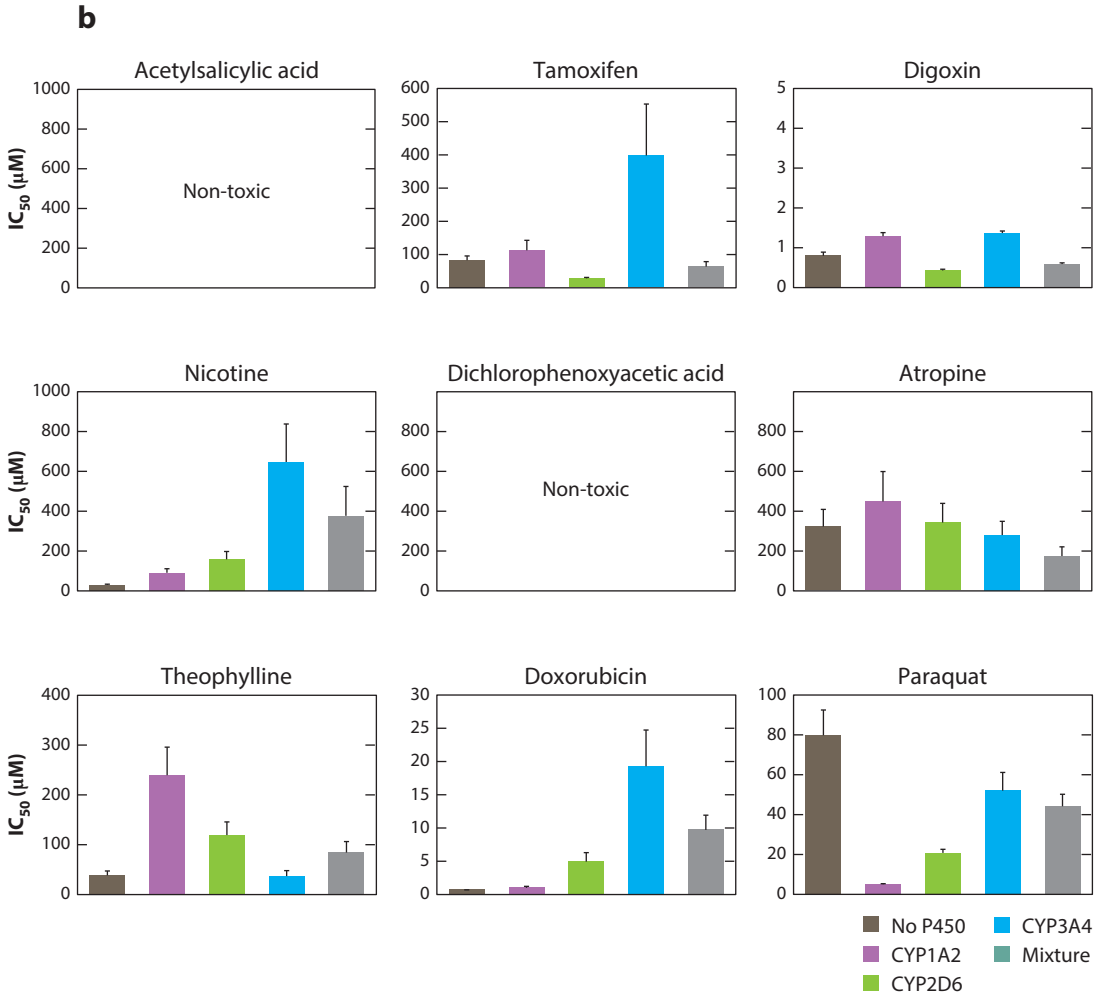

Figure 6

(*Continued*)

obvious that CMAs provide a significant improvement of throughput but some practical issues may have to be solved before the technology will be able to compete with well-established MTP-based assays (87, 88). The latter are the gold standard, provide significantly lower throughput, and also suffer from some technical shortcomings. The following list provides technical and practical issues that have to be considered and evaluated when MTP-based in vitro toxicology approaches are compared to CMA-based approaches with respect to their individual performances:

- The individual wells of MTPs are physically separated from each other. This separation prevents chemical crosstalk between the individual reaction chambers of the compounds being investigated but also molecules that are secreted by the cells and may affect the other populations (paracrine/autocrine signaling). Chemical crosstalk is still a concern in some CMA approaches.

- Not all viability assays that rely on the analysis of the cell culture supernatant for secreted molecules or indicators (e.g., MTT or LDH) are applicable in CMA-based assays, as the supernatant is shared between all μ-spots and the cell response cannot be assigned to one given spot or compound. Thus, the analytical readout for CMAs is confined to microscopic/imaging techniques in combination with staining of the most sensitive, nonmobile cell components or structures.
- MTP-based assays suffer from problems during compound washout. Complete washout requires aspirating the test solution completely, which leaves the cells at least for short periods of time uncovered by liquid. The remaining thin film of buffer evaporates quickly exposing the cells to the atmosphere or a rapidly changing osmolarity. Both instances cause trauma to the cells with unknown effect on cell physiology (89).
- CMA-based assays are considered more economical, as they require less incubation buffer, smaller amounts of costly assay reagents and lower cell numbers. Moreover, CMAs are less prone to evaporation problems (49).
- CMA-based assays with cell ensembles provide access to single cell information when high quality image analysis allows for an automated segmentation of the image to individual cells.
- MTP-based assays may suffer from their inherently large surface-to-volume ratio, which may lead to adsorption of the test compounds to the plastic surfaces, leaving the real compound concentration with some ambiguity.

In short, it is highly likely that CMAs will emerge as a mature assay technology to read cytotoxicity with unprecedented throughput assuming that the technical problems and challenges mentioned above will be solved in the near future. Recent progress in microfabrication techniques will assist and promote CMA development such that CBAs will be performed to a similar degree of parallelization as biomolecular interaction studies.

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