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Cell Microarray: An Approach to Evaluate Drug-Induced Alterations in Protein Expression

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Abstract: Designing reliable *in vitro* assays is crucial to obtain impactful results in oncology research. Here, we describe a histology-based method to evaluate the changes in biomarker expression after being subjected to several drug conditions and explore the mechanisms of therapy resistance in a chip-like tool, such as a cell microarray. This methodology has great potential in cancer research because we can evaluate a variety of cell culture conditions on a single microscope slide, enabling rapid screening of biomarkers using microscopic images of cultured cells. Indeed, the cell microarray presents several advantages over the Western blot option to evaluate protein expression in cell cultures, enabling visualization

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of the protein cellular localization and side-by-side condition comparison of the results. This chapter summarizes the main technical aspects of cell microarray construction, addressing the advantages and limitations, and its potential applications in the screening of biomarkers and tracking the phenotypic modifications on cancer cell lines after being exposed to different therapeutical conditions.

Keywords: biomarkers; cancer research; cell lines; drug tests; immunocytochemistry

INTRODUCTION

Tissue Microarray (TMA) is a histological system used to enclose several tissue cores in a single paraffin block (1). This approach is a powerful high-throughput screening method that enables the evaluation of tissue morphology and the molecular composition of several biological samples that can be visualized side by side on the same glass slide under a microscope (2, 3).

The current TMA technology was first described by Kononen *et al.* in 1998 (2, 4), nevertheless, the concept can be traced back to 1986 when Battifora designed the precursor 'sausage block' (5). This first approach consisted of 1 mm thick rods of multiple pieces of different tissues embedded in a single paraffin block, allowing staining of 100 or more tissue samples comprised in a single slide (5). In 1987, Wan *et al.* expanded and designed the array format allowing simultaneous analysis of multiple tissue samples; however, they faced interpretation challenges since it was problematic to identify the individual 'rods' (6). In 1990, Battifora and Mehta improved the method by developing a tissue block where samples were distributed in a checkerboard arrangement, making it readily identifiable after histological sections and under microscopic observation (7). In 1998, Kononen *et al*. addressed some limitations of this system and developed a technique for the rapid and accurate construction of TMAs, that allowed the examination of several histological sections simultaneously by arraying it into a paraffin block (2, 4). This is a cost-effective technique that allows the simultaneous evaluation of multiple formalin-fixed paraffin-embedded (FFPE) samples using current histology techniques (2, 8–10). Many modified versions of TMA have been implemented, including fully automated TMA systems (11–15). More recently, this approach was adapted to FFPE cell suspensions, renamed as 'cell microarray' (CMA) (16–22).

CELL MICROARRAY

The aim of CMA is to accelerate molecular profiling in cancer research by conducting large-scale studies while reducing experimental variables and saving scarce cell suspension samples. This high-throughput technology consists of a single paraffin block constructed by extracting cylindrical cores from different donor paraffin blocks and re-embedding them into a single recipient paraffin block at defined array coordinates (2, 9, 10, 23) (Figure 1).

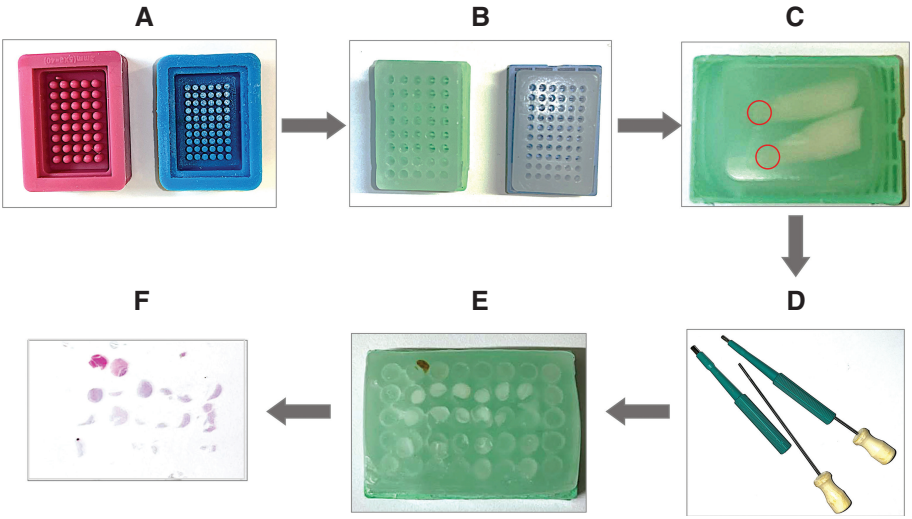

Figure 1. Cell Microarray construction tools. A, CMA molds used to produce recipient blocks with different core diameter sizes. **B,** Recipient paraffin blocks with different core diameter sizes. **C,** Donor paraffin block; each red circle represents a selected area of cell line suspensions cultured under a specific condition. **D,** Punch needles with different diameters sizes. **E,** CMA with all the cores from each donor block. **F,** H&E performed on a CMA slide. CMA, cell microarray; H&E, Hematoxylin & Eosin.

In 2005, Waterworth *et al.* and Ferrer *et al.* described CMA as an adaptation of TMA that incorporate multiple cell lines in a single array and demonstrated its usefulness to performing immunocytochemistry (ICC) studies in a single experiment (20, 21). Furthermore, Waterworth *et al.* showed that cultured cell lines can be successfully incorporated into CMA, with preservation of cell architecture (20). Ferrer *et al.*, showed that this system allows simultaneous analysis of multiple antigens in multiple cell lines under different experimental conditions; also it is suitable for long-term storage and a variety of techniques (21). With this approach, a complete cohort of samples can be analyzed simultaneously in a single slide under identical conditions, providing optimal rentability of the sample's resources (2). A CMA is particularly useful for the study of hematopoietic neoplasms (24) and serous effusion samples allowing the evaluation of DNA, RNA and/or protein expression in multiple samples simultaneously using many cytochemistry techniques, such as ICC, immunofluorescence (IF), *in situ* hybridization (ISH), proximity ligation (PLA), among other histology techniques (18, 19, 25–28).

Design and construction

For the construction of CMA, we selected a recipient block with a relatively low number of samples (35 cores) per block with a core diameter of 3 mm and used an Arraymold Manual Tissue Microarrayer© (IHC WORLD, LLC, MD, USA). Building a CMA with cultured cell lines requires the collection of cell suspensions,

fixing, processing, and embedding (22). An optimized number of cells per well was seeded under determinate conditions (e.g., different drug treatments) in cell culture plates and incubated at 37 ° C and 5% CO_2. At the end of the incubation time, cells were collected by directly and gently scraping the culture plates. As our goal was to evaluate the expression of several proteins, including cell adhesion proteins, we avoided the collection of cells by trypsinization, since this could modify the protein expression profile (24). After the removal of the supernatant, cells were washed three times with ice cold phosphate buffered saline and centrifuged at 1200 rpm for 5 minutes at room temperature (RT) (Figures 2A and 2B). Cell pellets were fixed in 10% (v/v) neutral buffered formalin (AppliChem, Darmstadt, Germany) for 1 hour with gentle agitation (Figure 2C). After fixation, cells were centrifuged at 2800 rpm for 5 minutes at RT, supernatant was discarded and cell pellets were embedded in liquefied Histogel™ (ThermoFisher Scientific, Waltham, Massachusetts, USA), according to manufacturer's instructions (Figure 2D). After centrifugation (4000 rpm for

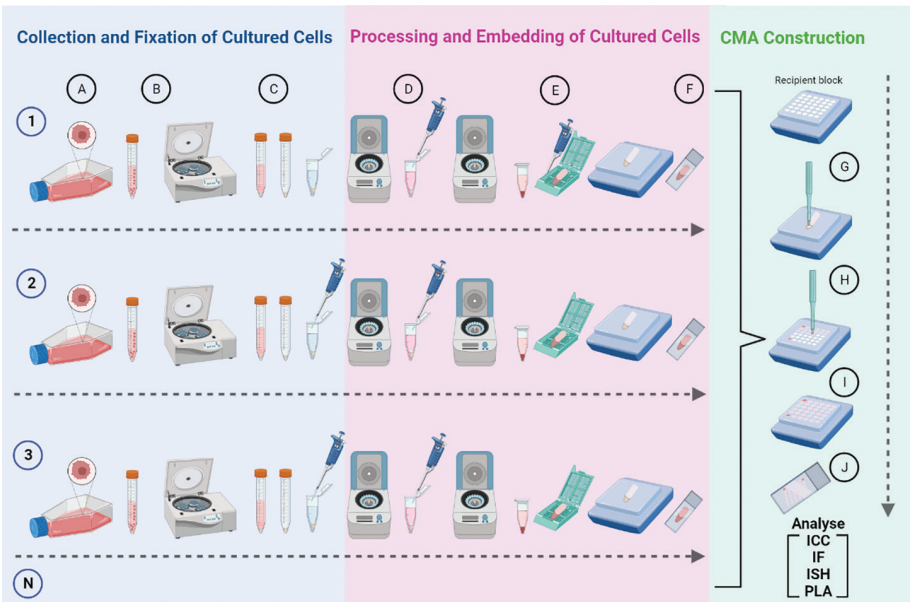

Figure 2. Graphic illustration of collection, fixation, processing, and embedding of cultured cells and CMA construction. A, Cells were cultured under specific conditions (e.g., drug treatment 1, 2, 3 - N). **B,** Cells were collected, centrifuged and **C,** fixed in 10% (v/v) neutral buffered formalin. **D,** Cells were centrifuged, and pellets were embedded in liquefied Histogel™ (ThermoFisher Scientific), **E,** placed in a histological cassette and submitted to standard histological processing. **F,** Cell areas were selected from H&E sections, and marked on the correspondent paraffin donor block. **G,** The cores were removed from the donor block, and **H,** inserted into the hole of the recipient block. **I,** The procedure was repeated to create a complete CMA block. The recipient block was slightly melted (37 ° C) to bind the cores to the paraffin block. **J,** Sequential sections of 2–4 µm were cut and adherent to a coated glass slide. The figure was created on BioRender.com. CMA, cell microarray; H&E, Hematoxylin & Eosin; ICC, immunocytochemistry; IF, immunofluorescence; ISH, *in situ* hybridization; PLA, proximity ligation assay.

1 minute at RT), cells were incubated at 4 ° C for 10 minutes, placed into a histological cassette and submitted to standard histological processing (Figure 2E). Briefly, histological cassettes were dehydrated in a series of alcohol concentrations [70% (v/v) – 95% (v/v) – 100% (v/v)], clarified with clear rite (ThermoFisher Scientific) and paraffin impregnation followed by embedding with liquefied paraffin at 60 ° C.

The most important step in creating a reliable CMA block is to identify the representative area using Hematoxylin and Eosin (H&E) staining to assess the morphology and cellularity of the samples (Figure F). Each donor block was sectioned at 2–4 µm thickness and stained with H&E for the evaluation of morphology and selection of the core area. In parallel, a recipient block is made by filling the chosen silicone mold (Arraymold Kit A, IHC World LLC, MD, USA) with liquid paraffin and a histology cassette on the top of the mold. The paraffin-filled recipient block is cooled on a cold plate to allow solidification, followed by unmolding of the recipient block. The CMA block is constructed by placing cylindrical cores extracted with dermal biopsy punch needles (diameter between 0.6 and 4.0 mm, Arraymold Kit A, IHC World LLC, MD, USA) from each donor block (Figure 2G and 2H) (29). The sampling process is repeated several times from different donor blocks until all the cores are placed in the recipient block (Figure 2I). A CMA is organized at a specific coordinate (XY guide) for sample identification; control tissue cores (e.g., normal and tumor tissues) are included for orientation and as positive/negative controls. The finished block is placed upside down on a clean glass slide and placed in an oven at 37 ° C overnight to facilitates fusion/adherence of the donor cores with the paraffin wax of the recipient block. In the next day, the homogenization step continues with a set of 3 cycles of RT and 37 ° C for 1 hour each and a final step in which the CMA block is heated at 60 ° C with a glass slide on the surface for a final homogenization step. After homogenization, the CMA block can be sectioned (2–4 µm thickness), placed on coated glass slides (Superfrost Plus®, ThermoFisher Scientific) and dried at 37 ° C to section adherence. The sections must be duly numbered and stained with H&E every 10 cuts for morphological control or to assess each sample size. The CMA block should be stored at 4 ° C covered by parafilm, and slides with CMA sections should be used immediately for ICC and hybridization techniques or stored at 4 ° C for a maximum of 3 weeks (Figure 2J).

Critical steps

There are several steps in CMA design and construction that are critical to deliver a high-quality and useful tool (30). In the donor block, it is necessary to perform an H&E staining prior to sample retrieval to accurately assess the areas that will be placed in the recipient block (8).

The type of mold used (number of cores/holes) is variable depending on the experimental design. Also, the size and the number of cores per sample or condition are also important issues, since they define the number of cores which must be placed into the recipient block. Another important point supporting the inclusion of more than one core from the same condition is the fact that cores can be lost during the sectioning and/or staining procedures (4, 30). Therefore, loss of arrayed cases can be minimized without compromising the efficiency of the array system by including at least two cores per condition. The arrangement of the cores

in the planification will depend on the type of study and how many cores are collected from each donor block. Whenever possible, it should be avoided to place cores from the same donor block side by side, to ensure independent results/replicates (30). In contrast to TMA, intra-sample heterogeneity is not an issue with CMA, but it requires an optimal cellular concentration/density to produce a donor block with a compact pellet and a satisfactory depth to maximize the number of sections that could be obtained. In our experience, the optimal pellet concentration necessary to obtain a satisfactory cell pellet for this purpose is between 1×10^6 and 5×10^7 cells per condition (24).

An organized insertion of the cores into the receptor block is another important point. It is essential that the CMA layout display an asymmetrical template so that it cannot be wrongly collected on the glass slide, causing an interpretation error. If the layout is asymmetric, even if it is collected a section up-side-down or flipped in the water bath after sectioning, when observed under the microscope, it is possible to identify the correct orientation (8). It is important to include 'orientation cores' at specific positions of the layout, such as paraffin and control tissue cores that can be identified macroscopically. For example, FFPE liver or spleen tissues show a dark color that allows identification of the position during sectioning, collection of sections from a water bath and microscopic analysis (30). Therefore, the control tissue cores serve two purposes, as 'orientation cores' and experimental controls (positive and/or negative). Alternatively, the orientation cores can be placed strategically outside the geometric margins of the CMA with the same objective of orienting the sections during the microtomy (30).

When ICC is performed, a border staining artifact can occur. Therefore, it is recommended to include 'protection wall' with control tissues or paraffin cores (30, 31). Additionally, it is better to disperse duplicate cores across the CMA to evaluate staining in different positions on the slide and include cores of different staining intensity to facilitate results interpretation (8).

Once all cores are placed in the recipient block, it is necessary to slightly melt the paraffin between the cores and the recipient paraffin block to make the homogenization. This step is critical and should be performed carefully to seal the paraffin block by gently running the surface of the microarray block over a glass slide on a hot plate, to melt the surface paraffin. In this step, it is very important not to squeeze the block against the glass slide to avoid block deformation (8).

Only experienced technicians cut the CMA blocks to avoid excess thinning and block misalignment. Additionally, sections should be picked up from a hot water bath with extreme care to avoid distortion, aligning the section parallel to the edge of the glass slide. A perfect alignment of the CMA on the glass slide will facilitate microscopic screening and the identification of core coordinates. It is recommended to perform a serial sectioning technique, numbered consecutive cuts/sections, and staining every 10^{th} section with H&E to assess the presence/absence of biological material in all the cores (8).

During microscopic observation, the position of each core should be checked to confirm the layout (30). As mentioned above, orientation spots (black/paraffin cores) and asymmetrically placed control tissue cores facilitate and ensure proper orientation. This control evaluation step must be done by multiple independent observers and supported using digital scanning tools to evaluate the results (32).

The applicability of CMA to evaluate drug-induced alterations

Translating new findings from basic science to clinical practice is a crucial step in cancer research. Chemoresistance in many neoplasms is a major clinical problem requiring further study and a greater understanding of the mechanisms involved in testing drugs alone or in combination to overcome resistance. Any investment in this research area will have a great impact in the search for more efficient treatments. Currently, there is a lack of reliable biomarkers to select patients that would benefit from different treatment strategies. Therefore, studying chemoresistance mechanisms and performing a drug screening in a large number of cancer cell lines treated with different drugs alone or in combination is crucial to find new predictive biomarkers (26). In drug testing, cell viability assays are used to expose cell lines or patient-derived cells to different treatments and concentrations to find the best treatment option (33, 34). After evaluating IC50 in each condition, it is imperative to find the molecular modifications induced by each treatment. Therefore, CMA enables researchers to study and evaluate different samples/conditions, arising as a cost-effective and time-saving methodology that can be adapted to evaluate resistance mechanisms in *in vitro* drug screening tests. In addition, the possibility of having many conditions in a single slide has the potential to accelerate anticancer drug efficacy studies, allowing the discovery of biomarkers capable of predicting therapy responses and to unveil the mechanism of action of new or repurposed drugs alone or in combination schemes. Furthermore, the molecular alterations disclosed through analysis of CMA using different cell lines could represent a pre-clinical step to further explore tumor phenotypes, the identification of new predictive/prognostic biomarkers, and the validation of therapeutic targets of newly discovered genes.

In our research group, we have created several CMAs using different panels of cancer cell lines, proving its usefulness in the context of different cell culture assays (33–36). Figure 3 shows an example of a CMA created by our research group, in which we placed cells in the same block/slide with and without Paclitaxel exposure. Two Paclitaxel resistant cell lines were established from parental OVCAR8 by continuous (OVCA8 PTX R C) and pulse (OVCAR8 PTX R P) exposure to a stepwise increasing concentration of Paclitaxel for 3 months (33). Then, all cell lines were treated with 10 nM Paclitaxel for 48 hours and all conditions were arrayed in a CMA block. Finally, we performed an immunocytochemical evaluation of P-glycoprotein (P-gp), which is normally located on the cell membrane, where it functions as a multidrug efflux pump that transports different substrates, including Paclitaxel (37). P-gp overexpression is the main mechanism of resistance to various chemotherapeutics, being responsible for pumping the drug out of cells, resulting in a low intracellular concentration of the drugs, leading to cancer cell survival (38). The immunocytochemical results were evaluated under a brightfield microscope (Leica DM2000 LED©,Leica Microsystems, Wetzlar, Germany) by two independent observers (MN and SR) that register the staining pattern (e.g., P-gp it is a membrane marker) and the percentage of cells stained (0%, 1%–10%, 11%–25%, 26%–50%, 51%–75% and 76%–100%). Our results indicate that long exposure to Paclitaxel (OVCAR8 PTX R C and R P) leads to an increase in P-gp expression (76% to 100% positive cells vs negative/residual expression) (Figure 3) (33). Here, the CMA methodology was used to study the

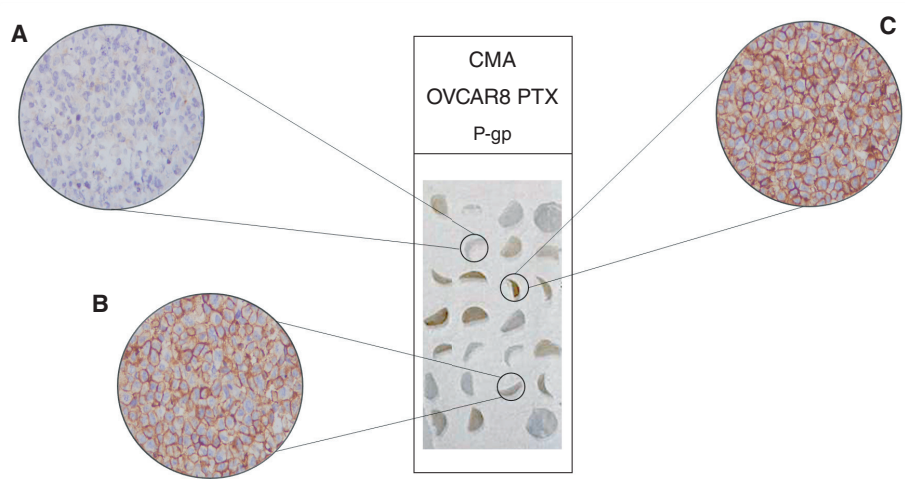

Figure 3. Representative slide obtained from a CMA block constructed from suspended cell lines. Representative immunocytochemical images for P-gp expression in OVCAR8 and OVCAR8 PTX R cell lines, after exposure to 10 nM Paclitaxel for 48 h. **A,** OVCAR8 (Control); **B,** OVCAR8 PTX R C; and **C,** OVCAR8 PTX R P (33). Figures are taken at 400x magnification, and the scale bar represents 100 µm. CMA, cell microarray; P-gp, P-glycoprotein.

molecular mechanisms responsible for Paclitaxel resistance and allowed to conclude that both OVCAR8 PTX R variants acquire a new phenotype that overexpresses P-gp (33).

Advantages and limitations

Several cell lines subjected to different culture conditions can be arrayed on a CMA, maximizing the number of experiments performed (39). Applied to different cell culture conditions, this system allows an increase in the number of studies per cell volume and decreases the number of cells needed per *in vitro* experiment since a small number of cells is needed for each condition (40). The grouping of all samples in a single block decreases the number of staining/protocols needed to generate a large amount of data in a relatively short time and in a cost-effective way (39). Also, CMA can produce hundreds of consecutive sections of different culture conditions and allows the storage of these samples in paraffin block for future use (1, 41). The methodology reduces the consumption of reagents and the volumes required to analyze several conditions in one batch on a single slide, compared to separate slides (42). Moreover, this allows for a more uniform analysis, since we can compare, on the same slide, the relative level of a specific protein's expression tested with the same experimental conditions (43). Thus, variables associated with different technician/researcher, dependent variables such as antigen retrieval, incubation temperature and time, washing procedure, and reagent concentration are the same for each sample arrayed on the slide saving the assay costs and human resources time (43). These types of microarrays are suitable for a wide range of cell culture experiments that can be further studied by histological techniques, including histochemical and immunological stains

with either chromogenic or fluorescent visualization, ISH, and PLA. CMA can be easily implemented in laboratories with access to histology techniques and with TMA construction expertise. A major advantage of this approach is the fact that the biologic resources enhance the testing of new biomarkers without the need to perform new cell culture experiments (35). Although building and sectioning a CMA can be challenging, it is an investment in the end, the time pays off by saving reagents, laboratory instruments and human resources needed to study a high number of sample conditions (8).

The major limitation of the CMA methodology is the size of the core that depends on the cellularity obtained in cell culture experiments (44). So, it is recommended to maintain a uniform depth of core insertion in the recipient block to obtain nearly the same number of CMA sections with all cores (8). Also, due to the small size of the cores, its loss may be more vulnerable to histologic processing/sectioning when compared to full sections. But this can be overcome with the inclusion of two or more cores for each condition per microarray and the use of high-quality adhesive slides (45).

CONCLUSION

The most important contribution of CMA is to provide a method that uses cellular suspensions to perform large-scale studies to explore DNA, RNA, and protein changes. Importantly, the CMA block can be archived for long periods of time and therefore readily available when new biomarker validation is needed. CMAs allow the analysis of several samples side by side on a single microscopic slide, minimizing the volume of reagents used and the biomarker evaluation in its cellular localization (e.g., nuclear, cytoplasm and membrane). Applied to drug-induced alterations, CMA can comprise multiple samples/conditions in a single block allowing the evaluation of the phenotypic kinesis of cancer cell lines before and after exposure to different drugs and the identification of putative therapy response biomarkers.

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