REVIEW

Demystified . . .
Tissue microarray technology

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Several "high throughput methods" have been introduced into research and routine laboratories during the past decade. Providing a new approach to the analysis of genomic alterations and RNA or protein expression patterns, these new techniques generate a plethora of new data in a relatively short time, and promise to deliver clues to the diagnosis and treatment of human cancer. Along with these revolutionary developments, new tools for the interpretation of these large sets of data became necessary and are now widely available. Tissue microarray (TMA) technology is one of these new tools. It is based on the idea of applying miniaturisation and a high throughput approach to the analysis of intact tissues. The potential and the scientific value of TMAs in modern research have been demonstrated in a logarithmically increasing number of studies. The spectrum for additional applications is widening rapidly, and comprises quality control in histotechnology, longterm tissue banking, and the continuing education of pathologists. This review covers the basic technical aspects of TMA production and discusses the current and potential future applications of TMA technology.

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Genomics, "transcriptomics", and proteomics are key words for a spectrum of new techniques, all of which generate extremely large sets/bodies of data in life sciences that require validation at the level of intact tissue. The conventional investigation of fresh frozen or paraffin wax embedded tissues is too expensive and time consuming to be applied to the characterisation of hundreds or thousands of genes or gene clusters associated with distinct tumour entities or other diseases. Thus, techniques that can facilitate research on a large series of tissues in parallel in a single experiment are required.
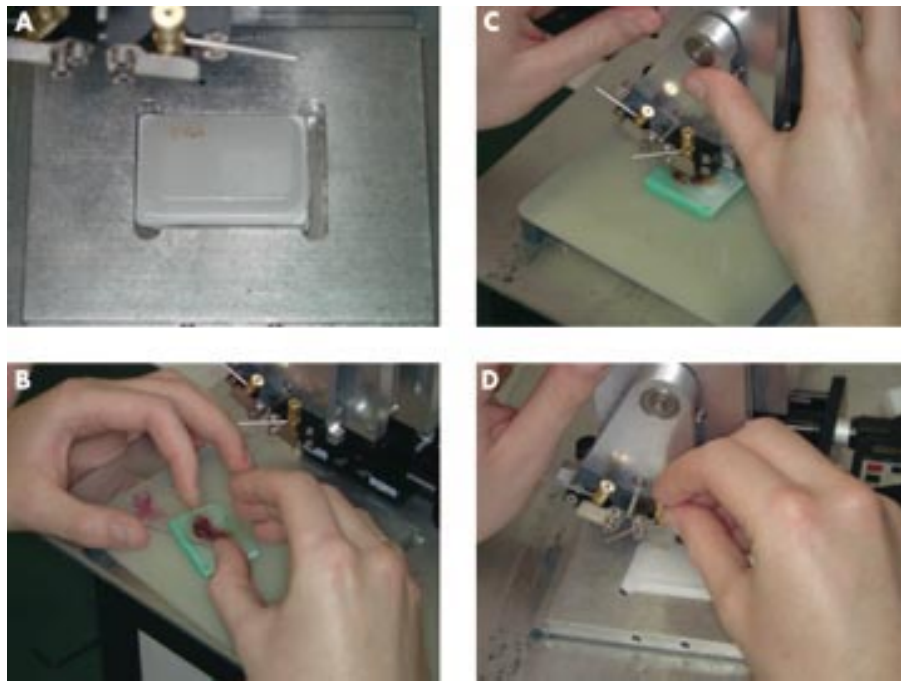
The idea of studying a large number of formalin fixed and paraffin wax embedded tissues simultaneously in a single histological section is not new. Various methods have been proposed, including the so called "sausage technique".[1] The major drawbacks of these previously described techniques were the limited number of tissue samples that could be included and the problems in identifying a distinct tumour or tissue sample in the big "sausage".[2] The microarray technique, described by Kononen *et al* in 1998, elegantly eliminated this drawback by the introduction of a high precision punching instrument, which enabled the exact and reproducible placement and relocalisation of distinct tissue samples.[3] Although the tissue microarray (TMA) technique was initially described and used for cancer research, the horizons for the use of this technique are now widening, and further applications have been proposed for routine pathology purposes.

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TMA CONSTRUCTION AND USE IN GENERAL

The construction of a successful TMA starts with the careful selection of donor tissues and precise recording of their localisation details.[4] All donor blocks and their corresponding haematoxylin and eosin stained slides have to be selected from the archives. The slides have to be reviewed so that suitable donor blocks can be selected and the region of interest defined on a selected paraffin wax block. All of those aspects of tumour classification, staging, and grading that cannot be extracted from the evaluation of an extremely small tumour sample have to be re-evaluated. Nevertheless, there are clear differences in the distribution of the workup time required for the individual steps in the conventional slide by slide and the TMA approach. With the use of TMAs, most of the work time is now focused on the preparation of the TMA, in contrast to the conventional approach, where a large proportion of time and materials is spent on sectioning and labelling. After initial review and selection of donor blocks, it is advisable to arrange and keep the relevant tissue donor blocks in the order that is represented in the TMA. This facilitates later reviews and the construction of arrays from the same donor blocks. It is also recommended that the array construction, which can be manual or automatic, is accompanied by the generation of a computer file, containing the tissue block coordinates and some of the basic patient data, or a patient identification number, if anonymity is

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Figure 1 Stepwise construction of a tissue microarray. (A) A paraffin wax core is removed from the fixed acceptor block. (B) The donor block is oriented with the help of the respective haematoxylin and eosin stained section. (C) The tissue core is removed from the donor block. (D) The tissue core is placed in the prepared hole in the paraffin wax acceptor block.

required. Although many authors prefer a conventional Excel file,[5] standardised worksheets are also offered online.[6] Every logical assignment on an array to relocate the punches may be useful. Nevertheless, most authors divide their TMAs into different rows designated by capital letters. These quadrants are further separated in a checkerboard order by letters and numbers. Further details are given in Bubendorf *et al*.[4]

Even though some authors prefer the use of proprietory techniques and instruments for TMA production,[7] most groups use the tissue array instrument by Beecher Instruments, San Prairie, Wisconsin, USA. This commercially available tool is still very similar to the prototype system used during the early development of the TMA technique. From our point of view, the limiting factor in the broad use of TMAs is the patience and skill needed by the person assembling the TMA, particularly when taking into account the remarkable price for this rather simple, mechanical tool.

Donor tissue core needles with varying diameters of 0.6 up to 2.0 mm are available. In our experience, the use of a tape section aid kit (Instrumedics Inc, New Jersey, USA) is not necessary, and can sometimes, when used improperly, give rise to false negative results, especially in fluorescent in situ hybridisation (FISH) analysis. Tissue cores are punched from a predefined region of a donor paraffin wax embedded tissue block. A haematoxylin and eosin stained slide arranged on the donor block surface is used for orientation (fig 1). Tissue cores are transferred to a recipient paraffin wax block, into a ready made hole, guided by a defined x–y position. This technique minimises tissue damage and still allows sections to be cut from the donor paraffin wax block with all necessary diagnostic details, even after the removal of multiple cores.

> "It is no major problem for a skilled technician to produce tissue microarrays from decalcified bone marrow biopsies"

Whereas the initial experiments concentrated on solid tumours, such as carcinomas or lymphomas,[8 9] it is also no major problem for a skilled technician to produce TMAs from

decalcified bone marrow biopsies (fig 2). Of course, there are technical restrictions that apply to the use of TMAs from decalcified arrays. Otherwise, examples for the use of FISH, chromogenic in situ hybridisation,[10] or RNA in situ hybridisation techniques (fig 3) are unlimited. With the generation of "frozen arrays", using fresh frozen tissue, even these last technical limitations will disappear.[11]

QUALITY CONTROL

Immunohistochemistry (IHC) is an established and widely used technique. Quality control in IHC is one of the major problems in daily practice. Even though IHC has been in use now for decades, there is still a high variability of intralaboratory and interlaboratory results, mainly because of interlaboratory differences in antigen retrieval, staining protocols, antibodies used, and in the interpretation of staining results. Therefore, approaches to guarantee a high level of quality are highly variable. The use of TMAs offers an alternative method of quality control for research and non-research purposes. This is urgently needed against the clinical background of an increasing number of therapeutic regimens based on IHC staining results for specific proteins (for example, the oestrogene receptor, erbB2, epidermal growth factor receptor, and c-kit).

TMAs can facilitate the standardisation of immunohistochemical staining procedures and interpretation by the introduction of external and internal quality assurances. In essence, two approaches to the use TMAs for these purpposes have been described.

The potential of TMAs in quality assessment for routine immunohistology has been demonstrated for several antibodies.[12–14] A major issue in constructing TMAs for control purposes rests in the selection of tissues with expression of a large number of different antigens. In addition to intact tissues, cell culture materials and cell lines may be used (fig 4). This also provides an approach to the semiquantitative evaluation of the expression of—for example, distinct oncoproteins—because the absolute expression levels of these proteins may be determined. Thus, a certain antigen profile
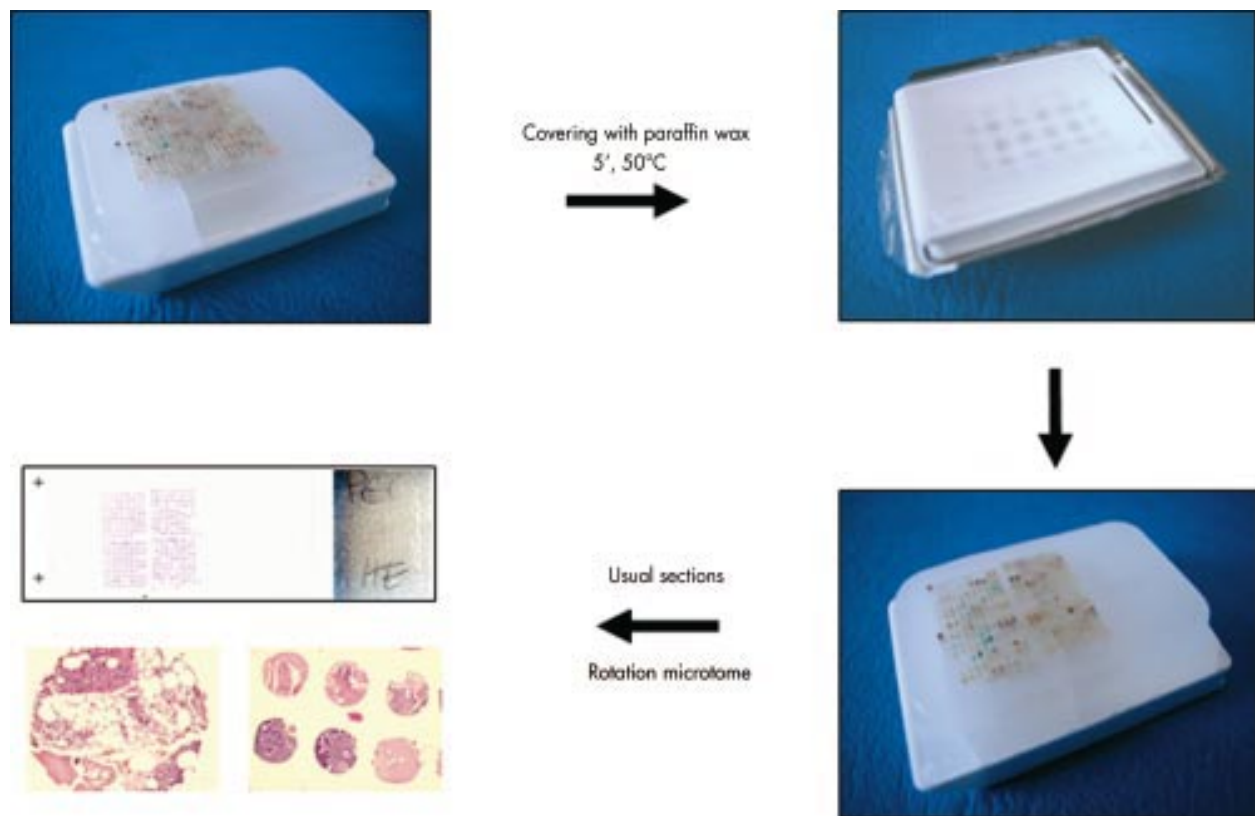
Figure 2   The resulting tissue microarray is covered upside down with melted paraffin wax and warmed in an oven for five minutes (50°C). The paraffin wax of the tissue cores and the acceptor block starts to homogenise, resulting in the final array block. Normal histological sections can be prepared from these blocks. Extra aids are not required, even if bone marrow biopsies are arrayed (lower left).

with defined end points for a positive or negative staining result can be defined. The requirement for such quality improvements has been demonstrated repeatedly by TMA based quality assessment studies. Up to 25% of all IHC stains differed significantly in different laboratories.[14]

To provide a high degree of intralaboratory reproducibility, another simple, but effective and reliable method for internal quality control in IHC has been described. With the use of internal control tissues in "mini-TMA format" some major problems in IHC could be reduced or even circumvented.[15] The test tissues and the tissue of interest are stained under the same conditions with the identical concentration of antibody (fig 5). In this particular setting, the very small tissue core diameter, often thought of as a major drawback of this

Figure 3   Example of RNA in situ hybridisation for procollagen obtained from a liver tissue microarray, as shown in fig 6D.

technique and therefore the whole mini-TMA system, is highly advantageous because it reduces the additional expense for antibodies and other reagents compared with external controls on separate slides. This approach also offers an alternative for the usually time consuming work to evaluate new antibodies and reagents. With the use of serial sections of an internal control TMA, variations in staining results will also be discovered immediately, regardless of whether they are caused by differences in reagents or variations in the staining procedure. The quality of IHC procedures may be improved by the consistent use of positive and negative control sections for all IHC incubations.

## ADVANTAGES AND DRAWBACKS OF PUNCHES WITH VARYING DIAMETER

The TMA approach has been criticised for its use of small punches of usually only 0.6 mm diameter from tumours with an original size of up to several centimeters in diameter, comprising areas of increased proliferation, apoptosis, matrix remodelling, necrosis, etc. Several experimental and clinico-pathological efforts has been made to reduce and even eliminate these concerns. Of course it cannot be overemphasised in this context that care in the composition of an array and a certain degree of redundancy is essential to minimise this effect, because the selection of different tumour areas should be oriented towards the requirements of the investigated tumour entity. The example of breast cancer might clarify this in further detail. The grading of breast cancer is dependent on the presence and number of mitoses.[16] Because this most important parameter of breast grading is evaluated mainly at the periphery of the tumour, breast cancer arrays focusing on tumour proliferation markers should be mainly composed of punches taken from the periphery of the original tumour.

Kononen *et al* clearly showed that the frequency of distinct prognostically significant amplifications, such as erbB2 or
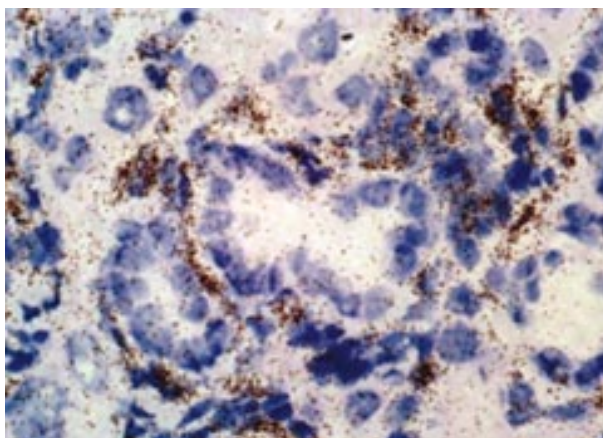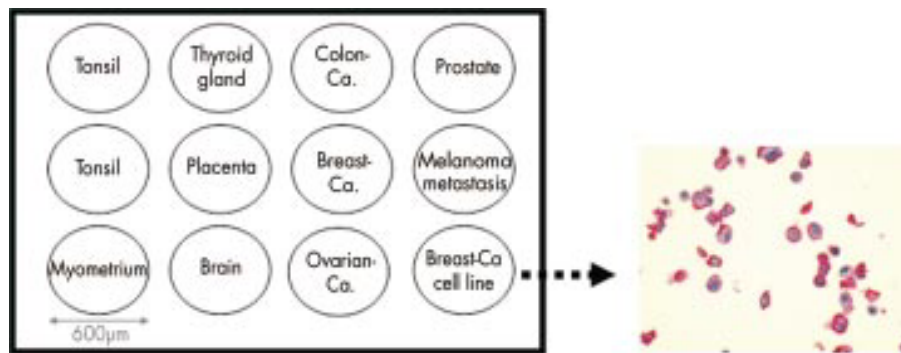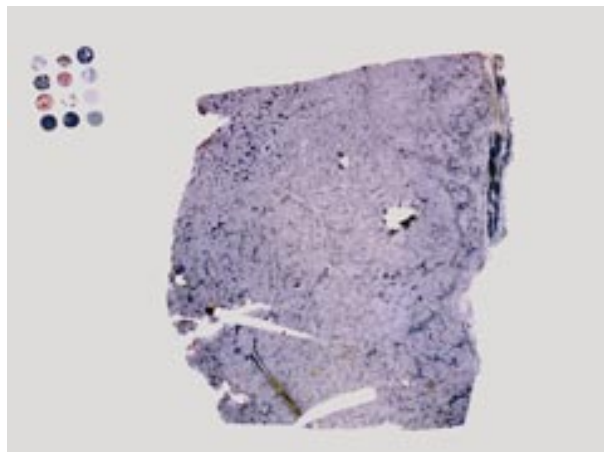
Figure 4 Composition of a tissue microarray for internal control purposes in immunohistochemistry. Different tissues can be used, according to the specific requirements of each laboratory. Next to paraffin embedded tissues cell line material with a known, quantifiable expression of distinct proteins can be used. On the right hand side, cells of the SKBR-3 cell line with a strong expression of c-erbB2 are shown. Ca., carcinoma.

cyclin D1, in series of invasive breast cancers assembled on a TMA was similar to frequencies described in the literature.[3] Similar experiences have been made with regard to the expression of different cytokeratins in breast cancer.[10 17] In addition, the influence of tumour phenotype on clinical outcome could be reproduced in an array composed of bladder cancer cases.[4] To guarantee the high quality and representivity of distinct tumour arrays, the introduction of a new array, designed to investigate perhaps new, so far unknown factors in carcinogenesis, should include at least some experiments, reproducing data described in the literature, as was done in the original description of TMA.[3]

> "The frequency of distinct prognostically significant amplifications, such as erbB2 or cyclin D1, in series of invasive breast cancers assembled on a tissue microarray was similar to frequencies described in the literature"

Other alternatives to circumvent these problems have been proposed, such as the use of larger punch needles of up to 2 mm diameter. At first glance this seems logical because the area available for evaluation is enlarged by a factor of 10. Nevertheless, for the use of TMAs in cancer research, no obvious advantage can be seen, because from our point of view when compared with the original size of a tumour with a diameter of up to several centimeters, an area of about 3 mm^2 (2 mm diameter) is hardly more "representative" than 0.27 mm^2 (0.6 mm diameter). In addition, the obvious disad-

vantage is that instead of several hundreds of tumours on a single slide/section, far fewer than 100 samples can be investigated at the same time. In our experience, these large punches also cause considerable damage to the donor and acceptor block using conventional paraffin wax blocks (an unfortunate example is provided in fig 6). The solution, if punches of 2 mm diameter are deemed to be superior to the conventional diameter, might be to use hand made (over sized) paraffin wax blocks as acceptor blocks (fig 6), because the increased thickness of these blocks might counterbalance the high tension forced by a 2 mm punch needle (our own unpublished experience). Despite the fact that these arrays might be suboptimal for cancer research, large punch arrays may be preferable for distinct areas of research. Research on complex tissues requires the simultaneous investigation of various regions in an organ. For example, in liver research, it is necessary to evaluate staining patterns of an entire acinus, including at least one portal tract and one central vein in the same tissue. With the use of 2 mm punch needles this requirement can be fulfilled in most cases (our own unpublished experience). Three punches of 2 mm diameter from liver tissue are sufficient to define the staining patterns in all the relevant anatomical regions. Larger needles might also be advisable for the investigation of small benign or malignant tumours in complex tissues. Lesions with a diameter of 1 mm or less (such as hyperplasias of the breast) might be missed or not punched as a whole entity, and will probably not be represented over the entire length of the cylinder.

Nevertheless, it is always necessary to stress the fact that the power of this technique has to lie within its ability to characterise and investigate large tumour series. However, the clinically reliable characterisation of single tumours/patients seems problematic because for erbB2 it was shown that six punches of a single tumour were required to reproduce the FISH results accurately.[8]

TMAs, GENOMICS, AND BIOMATHEMATICAL APPROACHES

Nevertheless, there are other lines of evidence indicating that punches of 0.6 mm may suffice in reliably defining tumour entities. With the use of TMAs in combination with gene chip technology or proteomic techniques, the amount of data generated with these methods becomes increasingly complex. To circumvent these problems and to trace down biochemical pathways or other regulatory mechanisms, biomathematical analytical procedures have been developed. The introduction of gene chip technology in cancer research has provided new insights in particular subgroups of breast cancer. Perou and colleagues demonstrated that in a series of sporadic breast cancers, different clinically relevant subgroups exist, with each subgroup being characterised by a specific RNA expression pattern.[18 19]
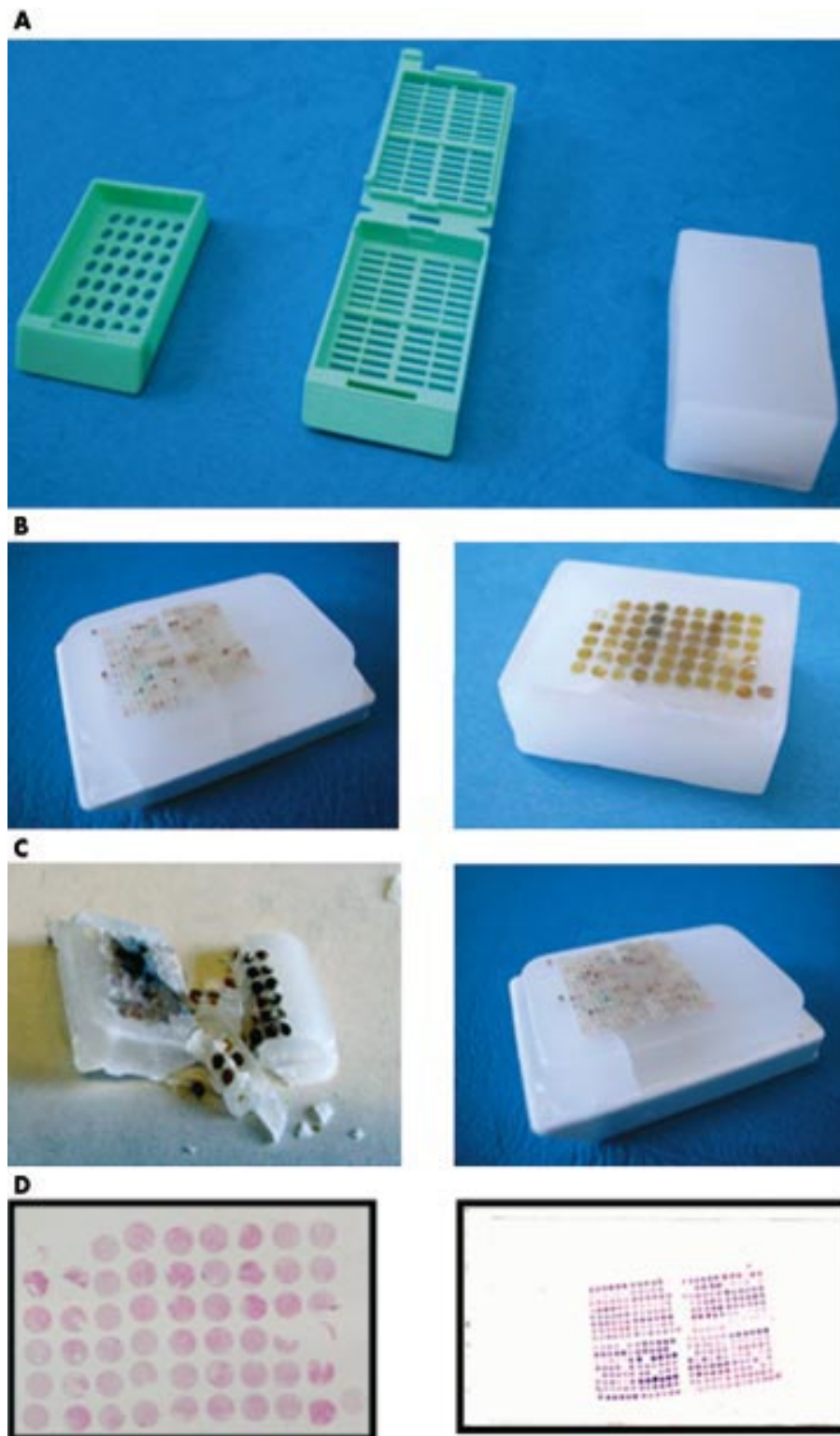

Figure 5 Example of a immunohistological section with an internal control on the slide in the form of a mini-tissue microarray, composed of 12 different tissues.

Figure 6 (A) Different acceptor blocks can be used for the generation of tissue microarrays. Whereas conventional histological cassettes (left and middle) are routinely used, individually formed paraffin wax blocks are superior if 2 mm punches are required. (B) Typical examples of tissue arrays. On the left, 0.6 mm punches have been used, whereas on the right a block with cores of 2 mm diameter is shown. (C) Problems of TMA blocks. A broken array is shown on the left. The array was composed of 2 mm diameter punches using a conventional cassette and the tension within the paraffin wax was too high. On the right side, the acceptor block shows a thin white line, which should be regarded as a first warning sign of increased tension within the paraffin wax, which might lead to array breakage. (D) Haematoxylin and eosin stained sections of liver tissue (2 mm diameter punches on the left) and cancer tissue (0.6 mm on the right)

Thus, they were able to define clusters of breast cancer cases characterised by the expression of high or low molecular weight cytokeratins, or by the expression of c-erbB2 (fig 7).[20] Using a similar biomathematical approach on a tissue array of invasive breast cancer cases, similar clusters could be identified based on immunohistochemistry alone (fig 8).[10] Therefore, TMAs in combination with other high throughput techniques not only tremendously speed up our ability to accumulate relevant data, but they have also contributed to an important change in our present understanding of carcinogenesis. Current concepts in

carcinogenesis largely rest on the idea of a gradual accumulation of genetic and epigenetic alterations in a small number of distinct genes thought to encode pivotal structures for distinct tumour entities. Because this approach has not satisfied initial expectations,[21 22] the interplay of the expression of distinct genes or gene groups, some of them thought to be of minor importance in the past, comes more and more into the focus of interest. Recent studies demonstrated that gene expression profiling harbours a higher clinical and prognostic potential for breast cancer or other tumour entities than single prognostic
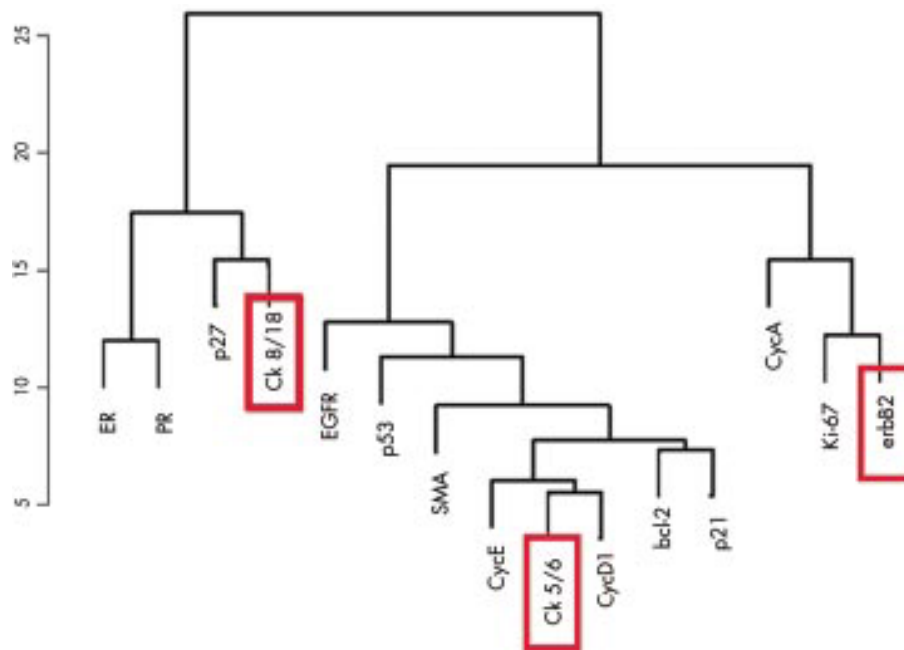
Figure 7 Hierarchial cluster analysis of more than 150 breast cancers, stained with 15 different antibodies. Three clusters defined by the expression of cytokeratin 5/6 (Ck5/6), Ck8/18, and c-erbB2 are shown.[10] The clusters are similar to results obtained from gene chip analysis.[18]

parameters.[23–25] Nevertheless, large tumour numbers are needed to establish reliable gene expression patterns at the protein level for diagnostic or prognostic purposes—once again the strength of TMAs.[26]

"Tissue microarrays in combination with other high throughput techniques have contributed to an important change in our present understanding of carcinogenesis"

TMAS AND CANCER CELL LINES

Experimental cancer research mainly relies on the investigation of complex cancer tissues, animal models, and cell lines.

Each of these fields is associated with advantages and pitfalls. In particular, cell lines are under constant criticism, because their potential is counterbalanced by the fact that only the tumorous subclones from a very complex tissue are present in cell culture. Therefore, the value of molecular data generated in cell lines is often hampered by their limited transfer to a clinical background. In this context, TMAs enrich the spectrum of laboratory tools immensely. Not only can the expression of genes under very defined conditions in cell cultures be verified per se by TMAs,[27] but using TMAs it will also be possible to correlate this—for example, with the presence of distinct extracellular matrix components or other factors that might influence the expression of a gene found to be important under cell culture conditions in vivo.
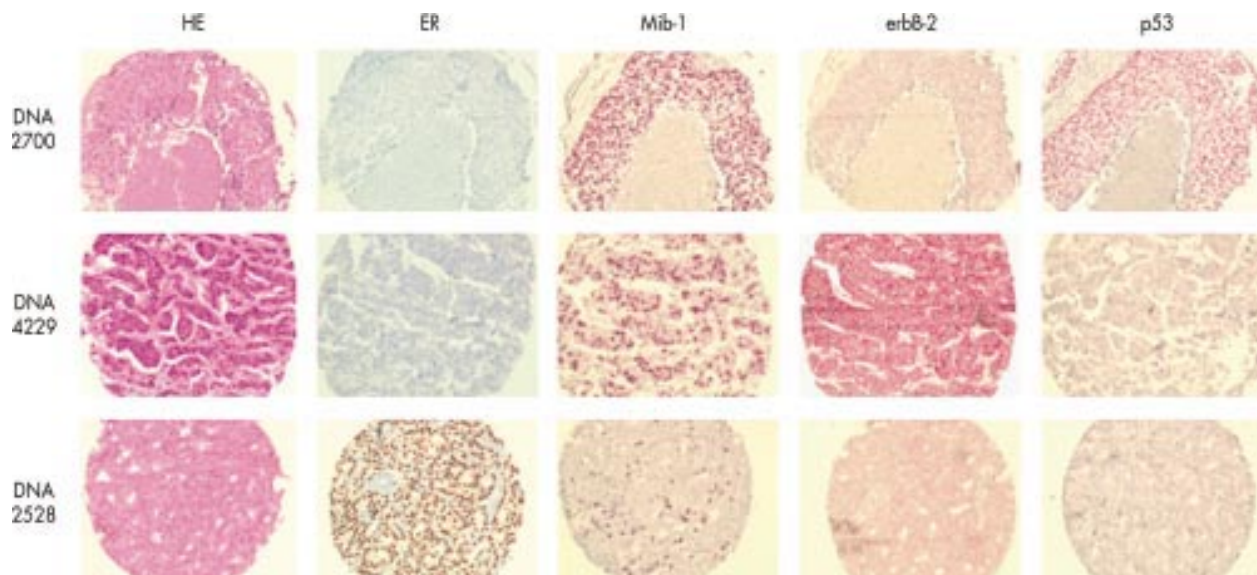
Figure 8 Tissue microarray technology allows a very detailed, immunohistochemical characterisation of large series of breast cancer cases. With the use of four different antibodies only, the heterogeneity of invasive breast cancer is clearly shown. E, oestrogen receptor; HE, haematoxylin and eosin.

FURTHER PERSPECTIVES

The era of tissue arrays has just begun. A multitude of different possibilities seems realistic, and some are already in use. For example, the use of "paraffin wax tissue banks" in pathology departments for the retrospective evaluation of new tumour markers for individual patients has been discussed to handle what some authors called the "titanic problem".[28] In detail, the speed with which new, prognostic factor dependent treatment modalities are generated and transferred to daily clinical practice and "predictive molecular pathology"[28] at the present time requires new methods to enable a retrospective patient tailored characterisation of these clinically relevant factors.[29] Despite these abovementioned drawbacks, this idea seems at the present state the most realistic and practicable one.

It will only be a question of time before TMAs find their role in educational purposes, as proposed by some authors.[4] Nevertheless, the major focus of TMAs at the present time is in the fields of cancer and non-cancer research.

In summary, the widespread use of TMAs will become an integral part of daily practice in research and routine clinical laboratories. With this clear perspective, "pathology" as an old, largely morphology based medical speciality will find itself in a central position within these new developments. With the background of archives of well characterised tumour cases, pathologists will be in the position to use the potential of TMA technology to present their well defined historical and current archives in an arrayed manner to the scientific community.

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