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REVIEW

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Tissue microdissection techniques in quantitative genome and gene expression analyses

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Abstract Current advances in quantitative genome and gene expression analyses allow precise molecular genetic fingerprinting of tumor tissues. A crucial factor for the reliability of the data obtained with these refined techniques is the use of morphologically well-defined cell populations. Microdissection technology has been developed to procure pure cell populations from specific areas of tissue sections under microscopic control. This review covers techniques of tissue microdissection in the context of commonly used methods of quantitative genome and gene expression analysis. The first part of the review will summarize the technical aspects of various methods developed for tissue microdissection. In the latter part, current applications of quantitative genome and gene expression analysis techniques employed in microdissected tissue samples will be described.

Keywords Tissue microdissection · DNA · mRNA · PCR · RT-PCR · CGH array · cDNA array

Introduction

Quantitative determination of DNA sequences and gene expression levels offers a powerful approach for the comparative analysis of normal and diseased, especially neoplastic, tissues. Remarkable progress has been made in recent years in the development of techniques for assessing DNA copy number and gene expression at the mRNA level. For instance, real-time polymerase chain reaction (PCR) allows the exact quantification of DNA or RNA [reverse transcriptase (RT) PCR] in tissue. More recently, microarray analysis techniques have been developed for quantitative large-scale analysis of gene copy number or gene expression. However, a crucial factor for the reliability of the results obtained with these advanced techniques is the use of morphologically well-defined cell populations.

Tissues are complicated three-dimensional structures, composed of different types of interacting cell populations. Since the cell population of interest might constitute only a minute fraction of the total tissue volume, the problem of cellular heterogeneity has been a major barrier to the molecular genetic analysis of normal versus diseased tissue. Thus, tissue microdissection represents one of the most promising techniques in molecular pathology, offering a link between morphology and molecular genetic analysis.

Tissue microdissection can be applied to routine tissue sections of both paraffin-embedded and frozen tissue as well as to cytological preparations. It enables the isolation of morphologically well-defined cells or cell groups that can be further processed for molecular genetic analysis. Microscopic control allows the definition of malignant or even premalignant cells and their dissection from the surrounding non-neoplastic tissue. The dissectates represent purified pools of morphologically well-defined cells with no or minimal contamination by non-neoplastic cells.

This review covers techniques of tissue microdissection in the context of commonly used quantitative genome and gene expression analyses. The first part of the review will summarize the technical aspects of methods developed for tissue microdissection, and the latter part will describe several current applications of quantitative genome and gene expression analysis techniques employed in microdissected tissue samples.

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Table 1 Overview of the most common microdissection techniques in molecular pathology. (*FFPE* Formalin-fixed and paraffin-embedded tissue, *FF* fresh-frozen tissue)

	Manuel tissue dissection	Laser microbeam microdissection (LMM)	Laser pressure catapulting (LPC)	Microdissection of membrane-mounted tissue (MOMeNT)	Laser capture microdissection (LCM)
Function principle	Procurement of large tissue areas using a sterile needle or a scalpel with/without an inverted microscope	'Cold-ablation' of unwanted cells using a UV laser (337 nm) Procurement of remaining cells with/without a micromanipulator under an inverted microscope	'Cold-ablation' of unwanted cells using a UV laser (337 nm) Procurement with 'non contact' laser pressure catapulting	Polyethylene foil as supporting membrane allows to cut out single cells or cell groups	Melting effect between selected tissue and a transfer film due to local heating by an IR laser (980–1,064 nm)
Minimum sample size	≅50–100 µm	<1 µm	<1 µm	1 µm	>7 µm
Preferential spectrum of use	Large and homogenous cell areas (>10^4 cells)	Small lesions (<50 cells), single cells, (also suitable for chromosome microdissection)	Single cells	Single cells or small cell groups (<50 cells)	Small cell groups (5–20 cells), large single cells
Specimen specification	FFPE, FF	FFPE, FF, fixed cells after cytocentrifugation, cell smear, chromosomes or metaphase spreads	FFPE, FF, fixed cells after cytocentrifugation, cell smear	FFPE, FF, fixed cells after cytocentrifugation, cell smear, chromosomes or metaphase spreads	FFPE, FF, fixed cells after cytocentrifugation, cell smear
Sample procurement	Manuel (sterile needle, scalpel) Micromanipulator	Computer-assisted micromanipulator	'Non contact' laser pressure catapulting directly into the sample tube	'Non contact' laser pressure catapulting directly into the sample tube	Thermoplastic transfer film
Preparation time	5–10 min for >10^4 cells	5–10 min for 1–20 cells	<3 min for 1–10 cells	<3 min for 1–10 cells	<10 s for 1–30 cells
Costs	Low	High	High	High	High
Advantages	Easy and quick method for large homogenous tissue areas	High precision, in particular for 1–10 cells	Minimalized risk of contamination under procurement	Rapid method to procure clear-cut cells with minimalized risk of contamination	Very rapid method to procure homogenous small cell groups
Dis-advantages	High risk of contamination Not suitable for heterogeneous tissue types Not suitable for small lesions (<50 cells)	Time-intensive	Danger of destruction of selected cells under procurement	Sophisticated preparation of tissue sections, exclusively for use membrane mounted tissue sections	Cost-intensive transfer tubes are necessary

Principles of tissue microdissection

Precision, avoidance of contamination and efficiency of the procedure are the most important parameters in tissue microdissection. The spectrum of techniques ranges from manual microdissection to single cell preparation based on laser- and computer-assisted systems. An overview of the most common microdissection techniques in molecular pathology is given in Table 1. In general, the isolation of premalignant or malignant lesions by microdissection requires a well-preserved histo- or cytomorphology and a trained pathologist.

Manual tissue dissection can be performed on routinely stained slides using 5- to 15-µm-thick sections placed on non-coated glass slides. Manual tissue dissection requires histologically homogenous malignant lesions, and the areas should have a diameter of at least 1 mm (Whetsell et al. 1992). Using a sterile needle or a scalpel the selected lesions can be procured (Perren et al. 1998).

The principle of *laser cutting* is a locally restricted ablative photo decomposition process without heating the direct environment of the laser beam (Srinivasan 1986). Within the diffraction limited focus of the laser beam obtained by a high numerical microscope lens a very high energy density is available, and if the pulse duration is shorter than the relaxation time of the biological material (range of µs) heat transfer is avoided (Greulich and Weber 1992). In this way a pulsed UV-laser microbeam can be used to cut or ablate stromal, inflammatory, or residual parenchymal cells surrounding the tumor cells in histological sections without destruction of genetic information of the remaining cells, as shown with different experiments (DeWitt and Greulich 1995). At the site of laser exposure and ablation, no amplifiable material is left behind (Becker et al. 1996). To retrieve the cells from the slide, a computer-controlled micromanipulator or conventional sterile needles are usually used to pick and transfer the cells into a tube for further molecular analysis.

Laser pressure catapulting (LPC) allows to catapult an isolated cell or cell group out of its surroundings with a single precisely aimed laser shot (Schutze and Lahr 1998). The ejected dissectates are either caught on a small piece of cover glass, or directly catapulted into the cap of a common PCR tube. The greatest advantage of this method is the procurement of the material in a 'non-contact' manner, which minimizes the risk of contamination.

For *microdissection of membrane-mounted native tissue* (MOMeNT) the tissue sections are mounted onto a 1- to 3-µm polyethylene foil (P.A.L.M., Bernried, Germany), which is attached to a slide by nail polish (Böhm et al. 1997). With a UV-laser microbeam tissue areas can be cut out with high precision. Combining this method with LPC one single laser shot makes it possible to catapult cell groups or even whole tissue areas of up to 1,000 µm in diameter. However, this method is more suited to procure small cell groups and single cells, if no or only minimal contamination by non-neoplastic cells is wanted. The MOMeNT technique implicates a special slide preparation with polyethylene foils, and excludes the use of routinely processed glass slides.

Laser capture microdissection (LCM) is helpful to select and procure cell clusters from tissue sections by use of a laser pulse. In LCM, a thermoplastic polymer coating attached to a rigid support is placed in contact with a tissue section. The polymer over microscopically selected cell clusters is precisely activated by a near-infrared laser pulse, and then bonds to the targeted area. Removal of the polymer and its support from the tissue section procures the selected cell aggregates for molecular analysis. Once the cells are captured, the DNA, RNA, or protein can be easily extracted from the isolated cells. The spectrum of application of this technique is wide, and it allows the fast procurement of histologically homogenous tissue areas or single cells (Simone et al. 1998). A great advantage is the well-preserved morphology of the transferred cells, which are attached to the removed polymer and can be readily visualized under the microscope. Ease and rapidity of use has been achieved by the commercial LCM microscope (Arcturus Engineering, Mountain View, Calif., USA; http://www.arctur.com). However, the focal spot of the melting laser cannot reach below 7 µm in diameter and there is no possibility to selectively destroy unwanted cells or tissue, neither adjacent nor within the selected area.

Tissue sources

Formalin-fixed and *paraffin-embedded biopsies* provide the main source of tissue for molecular analysis. Routine sections (5 µm) stained with hematoxylin and eosin are commonly used for tissue microdissection. Other histological stains such as methyl green or nuclear fast red may also be used (Burton et al. 1998). The sections can be mounted on routine glass slides for most techniques of microdissection. Immunohistochemical staining of the tissue sections prior to microdissection offers an additional phenotypic characterization (Fend et al. 1999a, b). It is helpful to increase the histo- and cytomorphology by covering the stained sections with a thin layer of xylene or 2-propanol which improves by wetting and refractive index matching the morphology on the unmounted slides. The xylene or 2-propanol evaporates quickly before cell procurement.

Sections from *fresh frozen tissue* can also be used for tissue microdissection (Hiller et al. 1996; Ponten et al. 1997). For an immunophenotypical characterization immunohistochemical staining procedures can also be applied to frozen sections (Fend et al. 1999a). However, the exact assessment of histomorphological details may be hampered in frozen sections.

The examination of *cytological preparations* from several organs such as the uterine cervix is well established for identifying premalignant or malignant cells. Routinely prepared cell smears stained with Papanicolaou can be used for microdissection and subsequent PCR analysis, even after storage of several years (Aubele et al. 1998). Other cell preparations, for example, cytospin samples, are also suitable to isolate cells or cell groups by microdissection.

DNA extraction from microdissectates

From the microdissected cells, DNA isolation according to standard procedures is possible if the samples contain at least 10^5 cells. However, the dissectates most often represent smaller samples. Thus, a simple one-step DNA preparation is recommended (Becker et al. 1996). The resulting DNA preparation is not 'clean', but is sufficient for PCR-based analysis.

PCR-directed amplifications require a careful control of reaction parameters, such as quality and quantity of the DNA template, to ensure reliable results. In contrast to the analysis of DNA that has been extracted from tis-

sue specimens without dissection, an accurate quantitation of template DNA obtained by microdissection before PCR analysis has so far been made difficult by the low amounts of DNA available for measurement. Although the amount of DNA extracted from microdissected cells can seemingly be estimated by counting the absolute number of dissected cells, significant deviations from the expected results may occur. It is obvious that all investigations aimed at the absolute quantitation of target sequences present within microdissected cells require a precise quantitation of the template DNA as an exclusive precondition. Serth et al. (2000) describes the quantitation of DNA after microdissection and extraction of cells with the PicoGreen fluorescence method. As limits of detection, about 24–40 diploid genomes, and, as limits of quantitative determination, about 73–120 diploid genomes were obtained. Furthermore, it was shown that formalin fixation as well as hematoxylin staining of frozen sections with Delafield's and Mayer's alaun or Weigert's iron hematoxylin before microdissection significantly diminishes the amount of extractable DNA and may lead to less reliable results, even of qualitative PCR analysis.

RNA extraction from microdissectates

RNA from microdissected tissue can be obtained by standard methods using commercially available RNA isolation kits. Microdissection by UV laser-based techniques must be carefully performed to eliminate all bystander cells because high copy mRNA transcripts from contaminating cells can produce erroneous results. Precipitative fixatives, such as ethanol and acetone, are believed to produce more RT-PCR amplification product than crosslinking fixatives such as formaldehyde (Goldsworthy et al. 1999). However, we found no differences in the qualitative expression of several genes in formalin-fixed compared to fresh-frozen tissue (Specht et al. 2001). For less than 10^5 cells, RNA amplification techniques should be applied. Using a T7-based RNA amplification in combination with cDNA microarrays, Luo et al. (1999) were able to demonstrate gene expression profiles from small cell samples of rat neurones. RNA was extracted from sets of 1,000 neurones obtained with LCM and linearly amplified an estimated 10^6-fold using T7 RNA polymerase (Luo et al. 1999). Alternate procedures for mRNA and DNA isolation from small tissue samples isolated by laser-assisted microdissection are described by Bernsen et al. (1998).

Genomic PCR

Alterations in gene copy number are one of the most important causes for deregulated gene expression and neoplastic transformation. Investigations of the pathogenic or prognostic significance of gene amplification require a reliable, sensitive, and objective method for the determination of gene copy numbers in tumor samples. The recent introduction of fluorescence-based kinetic PCR procedures offers a new tool for a very sensitive and accurate quantification of even minute amounts of nucleic acids. In principal a quantitative real-time PCR assay can be developed and validated for all loci in the human genome for which sequence information is available.

Lehmann et al. (2000) combined laser-assisted microdissection of tumor cells with the 5′-exonuclease-based real-time PCR assay. As a model system, amplification of the c-erbB2/HER-2/neu gene and the adjacent topoisomerase II alpha gene was determined in paraffin-embedded breast cancer specimens after immunohistochemical labeling and laser-based microdissection (LPC technique; P.A.L.M. laser microbeam system) of tumor cells. The high sensitivity of real-time PCR enabled the reliable and objective detection of even low-level amplifications in as few as 50 cells from archival tissue sections. Low-level amplification events were shown to escape detection unless tumor cells were isolated by microdissection. Furthermore, the authors demonstrated intratumoral heterogeneity by analyzing different areas of approximately 50 to 100 cells. This approach which combines immunohistochemistry, laser microdissection, and quantitative kinetic PCR allows morphology-guided studies in archival tissue specimens and enables the exact quantification of gene copy numbers in even small and precancerous lesions.

Transcriptional silencing of genes mediated by the epigenetic effects of DNA methylation at CpG-island-containing promoters has been well documented. Recent reports of silencing of tumor-suppressor gene expression by DNA methylation have emphasized the need for accurate, sensitive, reliable, and quantitative methods to measure levels of DNA methylation at specific gene loci. Xiong and Laird (1997) reported a quantitative technique called 'COBRA' to determine DNA methylation levels at specific gene loci in small amounts of genomic DNA obtained from microdissected paraffin-embedded tissue samples. Restriction enzyme digestion was used to reveal methylation-dependent sequence differences in PCR products of sodium bisulfite-treated DNA and it was shown that methylation levels in the original DNA sample are represented by the relative amounts of digested and undigested PCR product in a linearly quantitative fashion across a wide spectrum of DNA methylation levels.

Quantitative RT-PCR

The possibility to measure PCR product accumulation during the exponential phase of the reaction using fluorescent data has revolutionized not only DNA but also RNA quantitation. PCR and RT-PCR permit the quantitative determination of minimal starting quantities of nucleic acids down to at least 500 copies of a target sequence and are therefore particularly suited as downstream applications in combination with microdissection. The feasibility and reliability of real-time quantitative RT-PCR

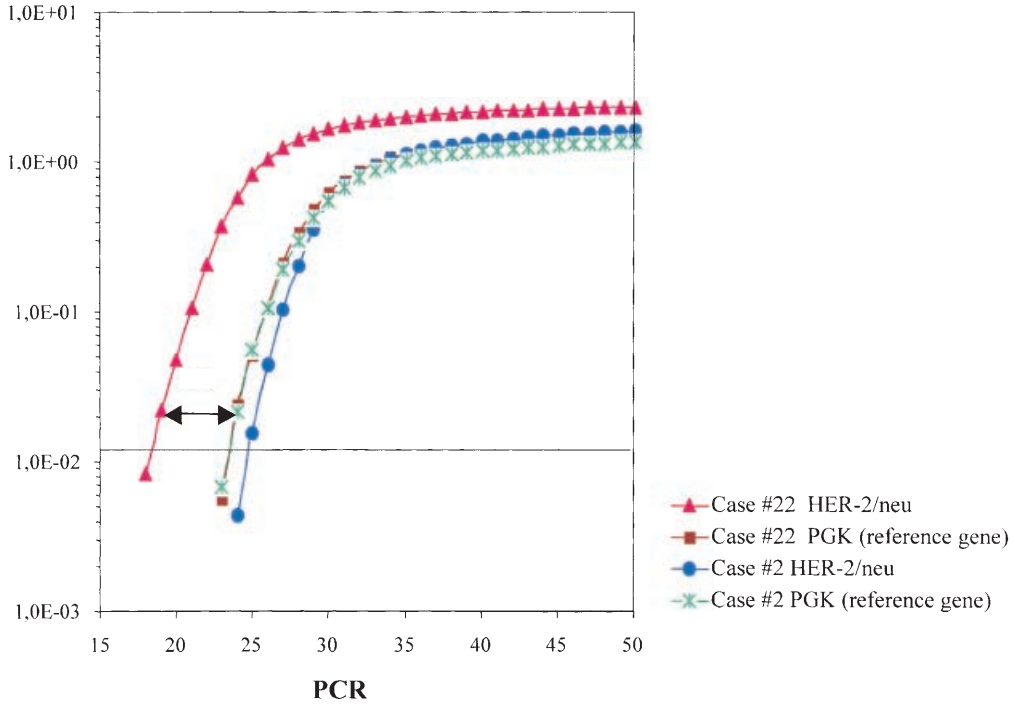

Fig. 1 Real-time TaqMan reverse transcriptase polymerase chain reaction (RT-PCR) amplification plots of HER-2/*neu* and PGK mRNAs in formalin-fixed cases of Barrett's adenocarcinoma (microdissectates of approximately 500 cells). *Case #22* displays normal HER-2/*neu* mRNA levels as compared to *case #2* (formalin-fixed, paraffin-embedded specimen) which shows strong overexpression of HER-2/*neu* mRNA indicated by a low Ct value in the amplification plot. The difference between the Ct for PGK and the Ct for HER-2/*neu* (ΔCt) corresponds to the relative expression level of HER-2/*neu*

coupled with microdissection of cells from frozen sections has first been demonstrated using a rat lung model. In this study, the authors could detect an upregulation of TNF-alpha mRNA in small clusters of microdissected alveolar macrophages (15 cells) of LPS/IFN-γ-challenged rat lungs, while TNF-alpha mRNA was nearly undetectable in macrophages selectively isolated from control lungs (Fink et al. 1998). Meanwhile, laser microdissection from frozen sections in conjunction with quantitative RT-PCR is becoming increasingly popular as a means to study gene expression and is now being used by several groups (Sgroi et al. 1999; Fink et al. 2000a, b; Xu et al. 2000). Very recently, it was demonstrated that quantitative RT-PCR can also be applied to study gene expression in microdissected tissue samples from archival formalin-fixed tissues, for an example see Fig. 1 (Goldsworthy et al. 1999; Specht et al. 2001). Specht et al. (2001) assessed the influence of several RNA extraction techniques, formalin-fixation, and laser-assisted microdissection on mRNA quantitation and demonstrated that expression level determinations from archival tissues were comparable to matched frozen specimens when using small target sequences in a range of 60–100 bp for real-time RT-PCR amplification. Furthermore, it was shown that mRNA quantitation could be reliably performed from as few as 50 microdissected formalin-fixed cells.

Thus, mRNA recovery and quantitative analysis is possible even from archival routine microdissected specimens, suggesting that these tissues can serve as a useful template for real-time RT-PCR analysis of a broad range of individual genes as well as newly developing high-throughput gene expression methodologies.

The targeting of specific cells may be difficult using routine morphologic stains. While immunohistochemistry can identify cells with specific antigens, exposure to aqueous solutions may degrade mRNA. To address this problem, Fink et al. (2000a, b) combined immunostaining and mRNA recovery using LCM and defined optimal conditions for cDNA amplification from immunodetected cells. Parameters that were systematically investigated included kind of fixation, antibodies and staining reagents, incubation and total processing time, and digestion with proteinase K. The authors presented rapid protocols for immunohistochemistry and immunofluorescence with total incubation times of approximately 25–40 min and 10–20 min, respectively, and suggested cDNA amplification without a preceding extraction step. Applying these protocols to oligocellular clusters containing approximately 20 cell profiles and nuclei each from lung and kidney tissue, the highest efficiency rates of mRNA amplification were obtained when combining short-term formalin fixation, reduction of antibody incubation time, application of immunofluorescence, and digestion with proteinase K.

Comparative genomic hybridization (CGH) microarray (matrix CGH) technology

The main limitations of chromosome-based CGH are that it is limited in resolution to 10–20 Mb, it does not provide quantitative information about gene dosage, and it is insensitive to structural aberrations that do not result in a DNA sequence copy number change. Replacing metaphase chromosomes as the substrate onto which aberrations are mapped with arrays of well-mapped cloned nucleic acid sequences can eliminate some of these limitations. The arrays are constructed using a robot to place

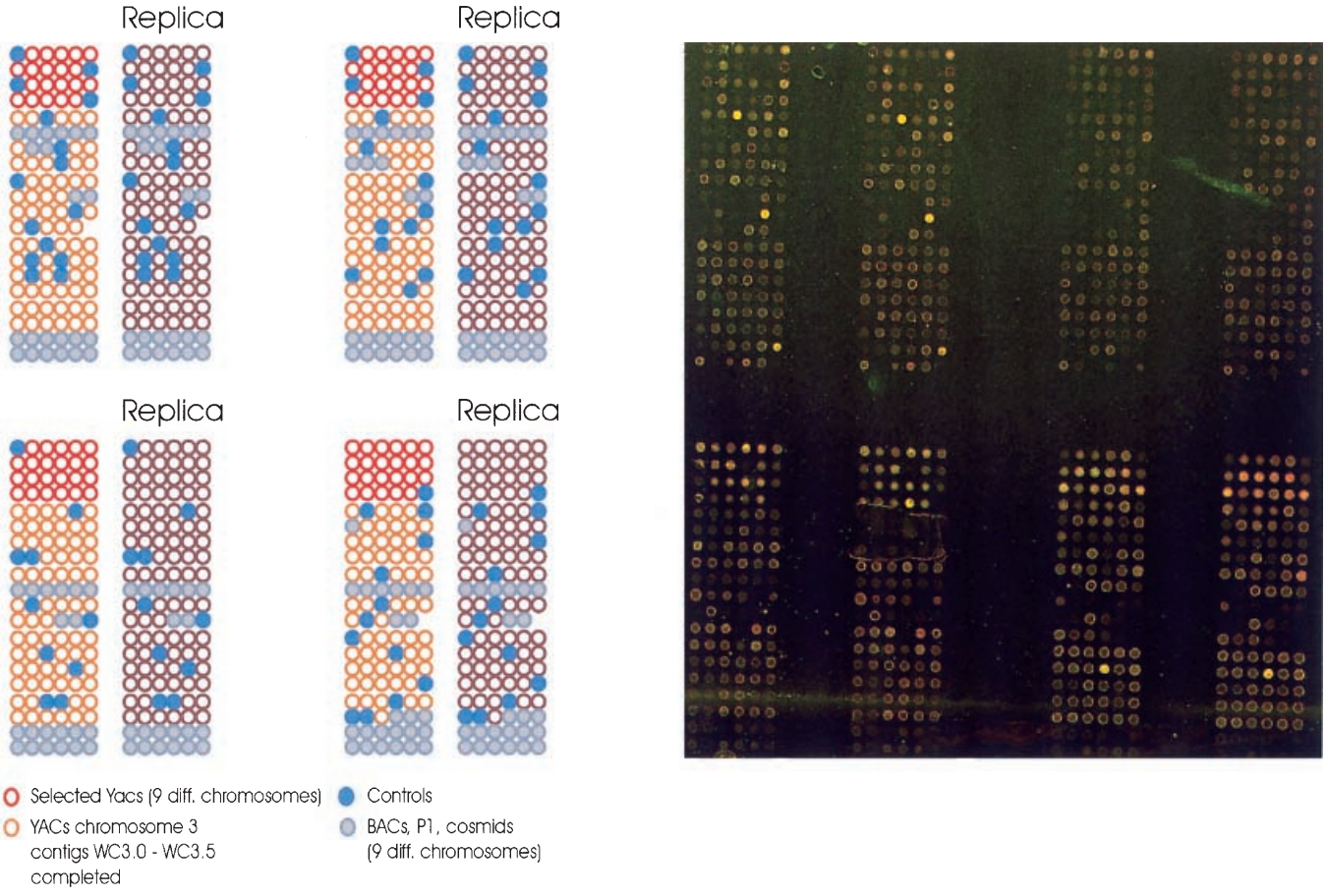

Fig. 2 Example of a comparative genomic hybridization (CGH) microarray experiment. *Left side* CGH microarray design (chromosome 3): 479 DNA probes and 479 replicas; 373 different ALU-PCR products from YAC clones, additionally 54 available BAC, P1, and cosmids, were spotted on glass slides. Five different 96-well plates of template DNA probes were used for this particular microarray design. Controls (52 probes of normal DNA) were dispersed randomly over the entire microarray. *Right side* Composite image of a differential hybridization generated using two different fluorescent dyes Cy3 (false color green) for labeling of tumor DNA and Cy5 (false color red) for normal control DNA. The genomic DNA used for hybridization was obtained from microdissectates from approximately 10,000 cells

clone DNA in high-density arrays on glass substrates. Array densities as high as 10^4/cm can now be achieved. Initial work involved CGH to arrays comprised of targets spanning >100 kb of genomic sequence, such as BACs (Pinkel et al. 1998), and recently STS-mapped YAC clones were used as targets achieving more than doubled coverage of the chromosomal region of interest (Fig. 2). In completion to common cDNA array studies this approach appears to be useful and clearly demonstrates that changes in genome copy number can be detected and mapped at a resolution defined by the genomic spacing of the clones used to form the array. Furthermore, CGH matrix array allows quantitative assessment of DNA sequence dosage from one copy per test genome to hundreds of copies per genome (Pinkel et al. 1998). The high resolution of CGH matrix array compared with chromosome CGH and the opportunities for quantitative aberration definition are apparent in genomic analysis, since the approach of microarray CGH has now been demonstrated in several laboratories (Solinas-Toldo et al. 1997; Pinkel et al. 1998; Pollack et al. 1999). Figure 2 presents a matrix CGH of a laser-microdissected breast cancer specimen obtained from formalin-fixed paraffin-embedded tissue. Most recently, CGH to cDNA arrays has been demonstrated (Pollack et al. 1999). cDNA arrays are attractive for CGH since they are increasingly available and carry a very large number of clones. However, the sensitivity of cDNA clone-based CGH for detection of low-level copy number changes is likely to be less than that for CGH matrix arrays based on YAC or BAC clone DNA.

cDNA microarray

The concept of expression profiling led to the development of robotic methods for arraying thousands of cDNAs on microarrays. These cDNA arrays that can be spotted on either nylon filters or glass are hybridized with labeled aRNA or cDNA to generate a molecular fingerprint of a specific cell type, disease state, or therapeutic efficacy. The highly parallel data acquisition and data analysis on cDNA arrays allows the exact determi-

nation of complex changes in gene expression. Apart from cDNA microarrays, several other methods have been devised to study gene expression on a large scale: cDNA subtraction, differential display, representational difference analysis, expressed sequence tag sequencing, serial analysis of gene expression, and differential hybridization on either high-density spotted nylon filters or glass. Profiles of gene expression obtained with all these techniques, however, are only reliable and meaningful if they can be assigned to morphologically identified pure cell populations. Until recently, the application of cDNA array techniques has been limited to mRNA isolated from millions or, at very best, several thousand cells thereby restricting the study of small samples and complex tissues. Since the total RNA content of mammalian cells is in the range of 20–40 pg and mRNA accounts for only 1–5% of the cellular RNA, any attempt at single-cell profiling must be capable of dealing with a total of 10^5–10^6 mRNA molecules. Non-amplified RNA from microdissected tissue samples has been used as a radioactive probe for cDNA arrays, however at least 5,000–50,000 microdissected cells are required for this type of analysis (Sgroi et al. 1999; Leethanakul et al. 2000). This problem can be overcome by the method of linear amplification. However, until now there are only few reports on successful combination of LCM, T7-based RNA amplification, and microarray technology. Luo et al. (1999) showed that RNA amplification is reproducible between individually LCM-captured cells. Furthermore, the authors demonstrated a differential gene expression between large- and small-sized neurones in the dorsal root ganglia. For this study, two sets of 1,000 large neurones and three sets of 1,000 small neurones were captured for cDNA microarray analysis. Using a similar approach, Ohyama et al. (2000) successfully analyzed differential gene expression from LCM-captured specimens of oral cancer employing 120,000 microdissected cells as starting material for T7-based RNA amplification.

Conclusion

Tissue microdissection is a prerequisite for the establishment of molecular genetic fingerprints of specific pathological lesions, in particular neoplasms. The ongoing improvement of the accuracy of microdissection techniques as well as the increasing sensitivity of various advanced molecular genetic methods will allow investigators to determine new specific changes in human cancer on the DNA or RNA level. In addition to the identification of new diagnostic and prognostic markers, this approach could lead to the establishment of individualized therapies tailored to the molecular genetic profile of a tumor. Thus, the combination of tissue microdissection and quantitative genome and gene expression analyses will have an enormous impact on molecular pathology.

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