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Review

Advances in isolation and characterization of homogeneous cell populations using laser microdissection

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Summary. The isolation and characterization of homogeneous cell populations are of great importance for the analysis of gene expression, because normal tissues contain various types of cells, and the differences in the populations of isolated cells exert significant effects on gene expression analysis. Researchers have attempted to develop methods for the isolation of homogeneous cell populations, such as flow cytometry and mechanical dissection. However, the recent emergence of laser-assisted microdissection has revolutionized the isolation of single-cell populations from solid tissues. With the help of a cutting laser, laser microdissection can isolate tissues (cells) of interest without contamination from surrounding tissues with the microscopic visualization field. By combining laser microdissection and subsequent microarray technology, several studies have resulted in the identification of disease-related genes. In this review, we summarize the principle of laser microdissection and provide several successful examples of target-gene identification using the conventional method combining laser microdissection and microarray. Next, we discuss the practical drawbacks of the combinational method, such as the need for a large number of cells and the disturbance of the relative abundance of transcripts during RNA amplification. We introduce our modifications to combined laser microdissection and microarray for detection of disease-related genes; the technique is simple, yet practical and accurate. Finally, versatile applications of laser microdissection, not only to transcript expression analysis, but also to other genomics and proteomics analyses are, also presented.

Key words: Laser microdissection, Microarray, System biology, RNA amplification

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Introduction

The isolation of homogeneous cell populations, and subsequent molecular analysis of uniform samples, have provided insights into physiology, disease pathology, and developmental biology. For example, the understanding of the physiological development of various leukocytes was possible only recently because of the ability to sort particular cell types by flow cytometry (Baumgarth and Roederer, 2000; Elewaut et al., 2003). Flow cytometry sorts cells based on a fluorescent tag on the protein expressed in a specific cell type, and allows the collection of several different cell populations simultaneously by multicolor fluorescence-activated flow cytometry (Baumgarth and Roederer, 2000). This knowledge led to the understanding of malignant leukocyte development (Williamson et al., 1987; Qiuping et al., 2003), which successfully led to the development of novel anti-cancer agents (Druker et al., 1996; Grillo-Lopez et al., 2001). However, this technique has limitations when applied to solid samples, in which the expression pattern would be disrupted by the dispersion procedure required.

Recently, laser-assisted microdissection methods have been developed to procure a homogeneous cell population from heterogeneous solid tissue samples (Emmert-Buck et al., 1996; Schutze and Lahr, 1998; Kolble, 2000). In this technique, homogeneous tissues are isolated using a laser beam, and target-cells are visualized under a microscope. Precise excision with the laser beam combined with the excellent optical capabilities of the microscope results in amplification of the target cell population with less risk of contamination. Three main platforms of laser-assisted microdissection methods exist, all of which begin with a heterogeneous tissue section mounted on a glass slide. In laser capture microdissection (LCM), a thin thermoplastic film is placed directly on the tissue section and an infrared beam cuts the film, which is attached to the cell of interest (Emmert-Buck et al., 1996). This positive selection is performed until all the cells of interest are

identified from the tissue section; then the target cells adhering to the dissected film can be placed in a container. The other two methods utilize an ultraviolet laser as the energy source for excision. While a laser catapulting microdissection (LCP) system collects samples by laser pressure (Schutze and Lahr, 1998), in laser microdissection (LMD), the dissected cells are simply trapped in a vessel through gravity (Kolble, 2000). In all cases, mRNA, DNA, or proteins can be purified from the isolated homogeneous cell population for downstream analysis. Each platform possesses advantages and disadvantages. For example, each system provides a different resolution of the isolating area, which is related to the wavelength of laser light used. While LCM with an infrared beam gives the minimum dissection area of 7.5 μm, the minimum cut size of the systems using an ultraviolet laser is less than 2.5 μm. Several reports have reviewed the detailed principles and compared the three methods (Cornea and Mungenast, 2002; Eltoum et al., 2002; De Preter et al., 2003).

In addition to the optical power of the microscope, laser microdissection can utilize the visualization methods developed for the identification of the cell types of interest. Visualization can be conducted with tissue sections prepared by conventional methods such as paraffin-embedding (Kim et al., 2003), histological stain (toluidine blue) (Miura et al., 2002) or immunohistological stain (antibody) (Lindeman et al., 2002). In some cases, especially when isolating RNA, adjustments to the standard protocol are needed to avoid the degradation of biological materials (Kim et al., 2003; Mouledous et al., 2003; van Dijk et al., 2003).

Recent advancements in functional genome technology, such as DNA microarray, enable the analysis of thousands of gene expressions simultaneously (Schena et al., 1995; Lipshutz et al., 1999). During DNA microarray experiments, RNA isolated from tissues/cells are hybridized to the glass slides that are fabricated with thousands of DNA oligonucleotides. Each oligonucleotide sequence corresponds to a unique mRNA sequence. Therefore, a particular mRNA from the tissues/cells binds with sequence complementation to the oligonucleotide probe. By quantifying the amount of mRNA binding to each probe, the expression of genes can be monitored with high throughput. Expression profiling of entire human or murine genes is possible now (Sridhar et al., 2003; Takahashi et al., 2003); the ability to profile genes from other species will soon be possible (Nobis et al., 2003; Suchyta et al., 2003). With these data, we can begin a systematic investigation into how molecules regulate biological processes (gene expression) in a manner similar to the way engineers manipulate circuits. This concept of "System Biology" could completely transform our current understanding of biological processes.

This review focuses on the application of laser microdissection to microarray technology and we introduce our modified method for combining laser microdissection and microarray to identify disease-associated genes, which overcomes current limitations of these techniques. Versatile applications of laser microdissection, not only to transcriptome analysis, but also to other genomic and proteomic approaches, are also discussed.

Role of laser microdissection in microarray analysis

Microarray analysis has become an invaluable technology for biological research, especially for pharmaceutical research; such as molecular target identification, in which gene expressions of normal and disease samples are compared to identify pathology-associated genes; pharmacology, in which diagnosis and prognosis are obtained by the pattern of gene expression changes; toxicology, in which the efficacy and adverse effects of compounds are determined by the expression pattern of RNA isolated from tissues exposed to the compound; and off-target activity identification, in which several pathways with altered expressions are analyzed to examine the selectivity of compounds. Despite progress in microarray technology, such as incorporation of the latest lithographical technology (Shoemaker and Linsley, 2002) and refined computer algorisms for statistical analysis (Hariharan et al., 2003), a persistent problem of gene expression analysis remains

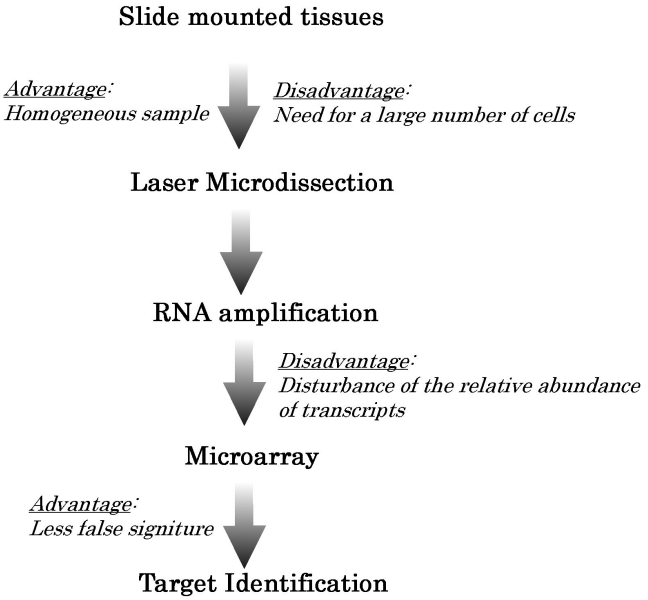

Fig. 1. Conventional method of laser microdissection analysis followed by microarray. A frozen-fixed tissue slide (not formalin-fixed) is used for the isolation of cells of interest by laser microdissection. The isolation of hundreds of cells and the amplification of RNA are required for the subsequent microarray analysis. After microarray hybridization followed by data mining with the appropriate algorism, the genes of interest can be identified. The conventional method has advantages, including the ability to accept homogeneous starting material, and disadvantages, including the need for a large number of cells and the risk of disturbing the natural transcript ratio.

the lack of purity of the starting tissue/cell material (Liotta and Petricon, 2003; Manfred et al., 2003). Analysis and interpretation of profiling data is very difficult when using complex starting material such as tissue samples that contain several, or in some cases, tens of distinct cell types (Liotta and Petricon, 2003). To overcome this limitation, laser microdissection is chosen for microarray analysis to amplify a particular cell type (Kondo and Raff, 2000; Miura et al., 2002). A flowchart of conventional analyses combining microarray and laser microdissection is shown in Figure 1. First, targeted cell populations in certain tissues are separated by laser microdissection from slides mounted/stained on glass. Next, RNA is isolated and amplified as needed for the analysis of the microarray. Because relatively large amounts of mRNA (10~100 μg) are required for each microarray experiment, several rounds of mRNA amplification are often associated with this step, followed by hybridization and data-mining processes. Based on this scheme, several studies have reported the successful identification of marker genes, pathology-associated genes, or cell-type-specific transcripts. Examples include identification of gene clusters that determine metastatic characteristics of small cell lung cancer (Kakiuchi et al., 2003), potential chemotherapy targets in breast cancer (Ma et al., 2003) and gene clusters distinguishing smokers from non-smokers and survivors from non-survivors five years after surgery for lung adenocarcinoma (Miura et al., 2002).

Compared to the whole-tissue approach, data obtained from the microdissection approach theoretically represent the authentic characteristics of the target-cell population. However, two major disadvantages are inherent in the process; the amount of labor involved in the microdissection processes and the necessity for mRNA amplification processes. Laser microdissection requires a large number of dissected tissues/cells (Glasow et al., 1998). For each microarray experiment, more than 10 μg of total RNA is necessary to obtain reliable data sets, in contrast to the 1.0 pg of RNA contained in a single cell. Preparation of a large number of cells by laser microdissection is very labor-intensive, and the possibility of sample degradation or contamination increases with each dissection. Even though a relatively large number of cells is collected by laser microdissection, the amplification of RNA extracted from the separated cells is an inevitable step. The amplification process can disturb the original relative abundance of various lengths of RNA, because shorter RNA types are usually more amenable to amplification (Nygaard et al., 2003). Several methods, including those based on RT-PCR or RNA amplification, have been developed for the accurate and non-biased amplification of transcripts (Wang et al., 2000; Baugh et

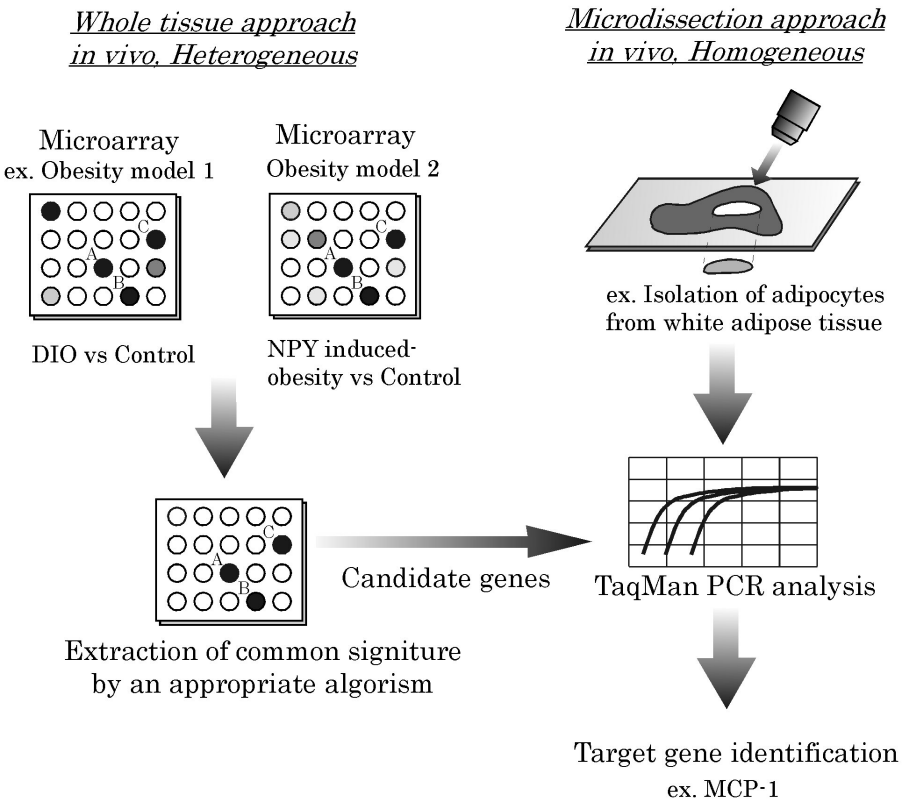

Fig. 2. Combination of whole-tissue and microdissection approaches. To overcome limitations of the whole-tissue and microdissection approaches, both approaches were combined. Firstly RNA from whole tissue was subjected to microarray analysis. To reduce false positive genes, several tissues from independent disease models were used. Common signatures, indicating candidates of disease-related genes, were analyzed for expression in microdissected homogeneous samples. The strategy was used to identify obesity-associated genes. DIO: diet induced obesity, NPY: neuropeptide Y, MCP-1: monocyte chemoattractant protein-1

al., 2001; Zhumabayeva et al., 2001). However, distinct differences exist between amplified and non-amplified samples in current protocols (McClintick et al., 2003). Therefore, a comparison between two samples should be conducted. For this scheme of laser microdissection and RNA amplification, approximately 1,000 cells are required for the subsequent microarray analysis (Glasow et al., 1998).

Combination of whole-tissue approach and microdissection approach

To circumvent problems of conventional microdissection, e.g., the necessity for large cell numbers and an RNA amplification step, we developed two strategies, as illustrated in Figures 2 and 3. Using these approaches, we successfully identified two disease-related transcripts using a small number of cells for laser microdissection (~100 cells) and no RNA amplification step for the microarray (Kobayashi et al., 2003; Takahashi et al., 2003).

Figure 2 illustrates the first strategy: a combinatorial method of whole-tissue profiling and candidate gene microdissection, which was applied to elucidate obesity-related transcripts as an example (Takahashi et al., 2003). Obesity mouse models were established, and microarray expression profiling was performed with RNA samples isolated from 'whole' white adipose tissues of obese mice and compared to control mice. The goal of this experiment series was the identification of obesity-related genes associated with obesity-related comorbidities such as atherosclerosis; therefore, we focused on the expression changes in adipose tissue, particularly in adipocytes. To reduce the number of candidate genes analyzed downstream, two different rodent models were employed to identify common signatures among different models. After the statistical analysis, several candidate pathophysiology genes were identified. During laser microdissection, we isolated homogeneous adipocytes from white adipose tissue (e-WAT). Adipose tissues consist of several different cell types such as adipocytes, vascular endothelial cells, smooth muscle cells, fibroblasts, mast cells and macrophages. The RNA extracted from the microdissected adipocyte sample was used for quantitative RT-PCR to investigate the expression level of the candidate genes listed by microarray analysis. Several dozens of genes can be quantified by RT-PCR.

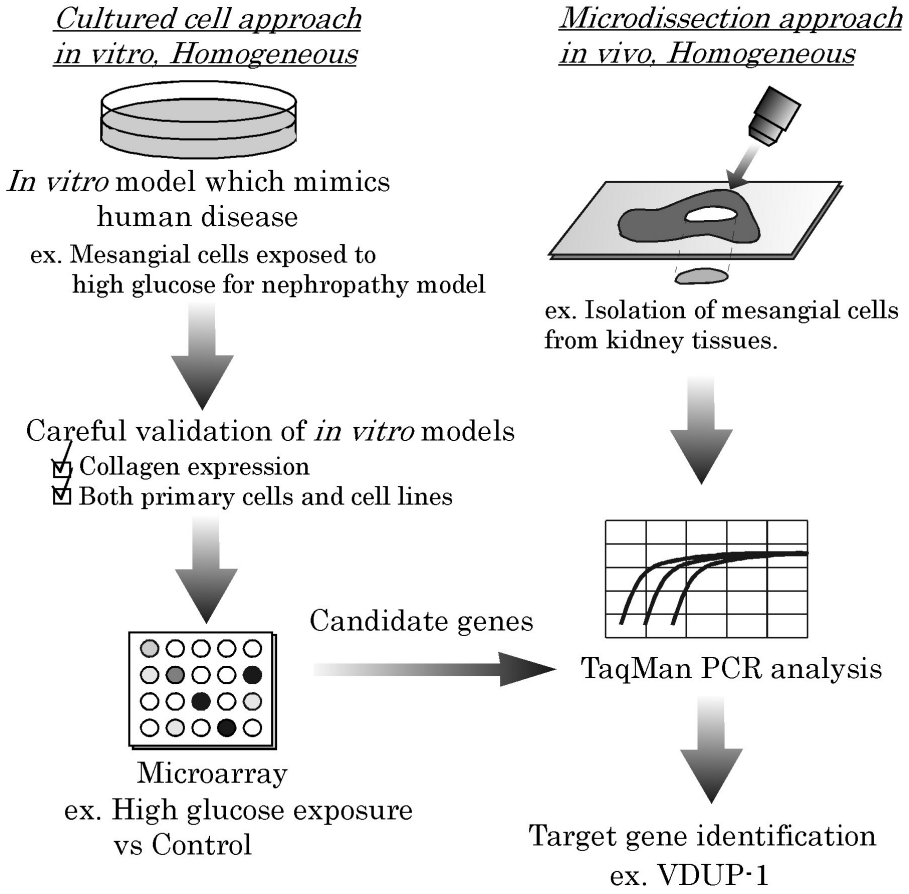

Fig. 3. Combination of *in vitro* and microdissection approaches. To overcome limitations of the *in vitro* model and microdissection approaches, both methods were combined. First, the *in vitro* disease model is established. Next, careful validation of the model is performed. Then, RNA from cells grown *in vitro* is subjected to microarray expression analysis to detect candidate genes. Finally, expression of the candidate genes is investigated in the microdissected samples to identify the disease-related genes. This strategy was used to identify the nephropathy-causative gene. COL: collagen, VDUP-1: vitamin D3 up-regulated protein-1

Through quantitative RT-PCR, we identified monocyte chemoattractant protein-1 (MCP-1) as an obesity-related gene, and confirmed its function in obesity by using standard pharmacological techniques (Takahashi et al., 2003). This strategy involves the use of laser microdissection for the verification of microarray data obtained from the whole tissue approach, which eliminates the requirement for large cell numbers and an RNA amplification step for laser microdissection.

Combination of *in vitro* cell culture and microdissection approach

Our second strategy, shown in Figure 3, combined *in vitro* cell culture and *in vivo* laser microdissection for detection of disease-related genes, exemplified by a search for diabetic nephropathy genes (Kobayashi et al., 2003). Diabetes is associated with several serious complications including diabetic nephropathy, in which renal mesangial cells produce a large amount of extracellular matrix leading to renal failure. The lack of access to human pathological samples, especially samples at all stages of disease development, required the use of an *in vitro* model of diabetes for the analysis. Human and mouse primary mesangial cells were exposed to a high glucose medium *in vitro* to mimic diabetic conditions. RNA extracted from the cultured cells was used in DNA microarray analysis to identify expression signatures for high glucose exposure. To validate relevance of the expression changes *in vitro*, a murine model of diabetic nephropathy, streptozotocin-induced diabetic mice, was used. We performed quantitative TaqMan PCR on laser microdissected samples from the homogeneous mesangial cells isolated from renal tissues of the diabetic mouse model. Using this approach, we identified the vitamin D3 up-regulated protein-1 (VDUP-1) as one of the causative genes for the accumulation of extracellular matrix in diabetic nephropathy (Kobayashi et al., 2003).

Both strategies described possess two characteristics: 1) Starting material for the microarray analysis is not a laser microdissected sample, but whole tissue or *in vitro* cultured cells and the experimental conditions are carefully designed to minimize false positive results from the microarray data (combination of several different disease models, both from the same or different species); and 2) laser microdissected homogeneous samples are used to validate expression levels of a limited number of genes selected from microarray analysis. Because the amount of RNA isolated by laser microdissection was sufficient for several rounds of analysis, there was no need for RNA amplification, and a realistic number of dissections were performed for each experiment.

Versatile application of laser microdissection

Analysis of biological phenomena in single cell lineage/populations is a universal desire in all fields of molecular biology. Hence, it is not surprising that laser microdissection has attracted attention in fields such as genomics and proteomics. We now discuss potential applications in the areas of research that benefit most from laser microdissection technologies (Fig. 4).

Application to proteomics

Similar to microarrays in genomics, two-dimension polyacrylamide gel electrophoresis (2-D PAGE) is recognized as a standard analytical platform in proteomics. Samples isolated by laser microdissection have been used for 2-D PAGE analysis (Seow et al., 2001; Craven et al., 2002; Zhou et al., 2002); however, they are limited by low sensitivity and quantitation ability for proteomics. The 2-D PAGE experiments usually require the collection of more than 50,000 cells by laser microdissection, making it laborious to use laser-microdissected samples as a starting material. The recent application of LC-MS/MS to proteomics has enhanced the sensitivity of protein identification; this can be combined with isotope-coded affinity tag (ICAT) technology (Gygi et al., 1999) to further enhance sensitivity and quantitative ability of protein identification by LC-MS/MS. With sequential application of ICAT and LC-MS/MS, one-fifth of the starting material required for conventional 2-D PAGE is adequate (Li et al., 2004). Other imminent advancements in LC-MS/MS technology will allow this technology to replace 2-D-PAGE analysis in the near future. Interesting applications of laser microdissection to protein array or MALDI have also been reported recently (Chaurand et al., 2003; Grubb et al., 2003).

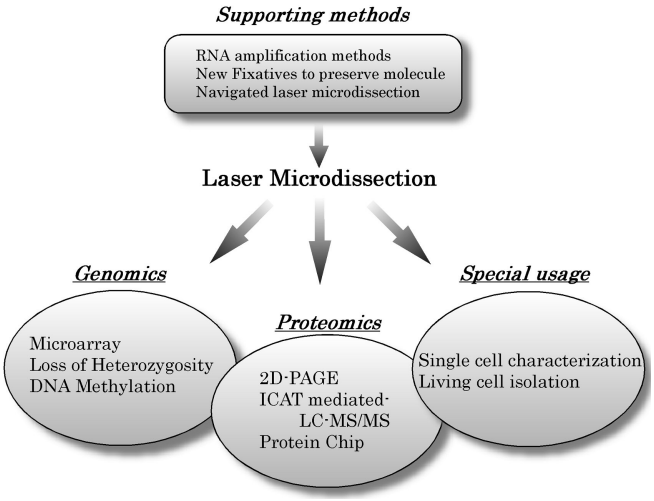

Fig. 4. Versatile application of laser microdissection. Laser microdissection, with the help of supporting methods such as RNA amplification and new fixative, navigated LCM, can be applied to the fields of genomics, proteomics, and other medical disciplines.

Application to genomics

In addition to expression analyses such as transcriptomics and proteomics, laser microdissection is also used in fields of genomics (Shen et al., 2000; Cui et al., 2001; Keohavong et al., 2004; Tuhkanen et al., 2004). Studies on DNA methylation, which inactivates a gene through chromatin modification and transcriptional inactivation, also utilized samples isolated by laser microdissection. For example, analysis revealed that the promoter region of the caveolin-1 gene was hypermethylated in prostate cancer compared to matched normal samples using laser microdissection (Cui et al., 2001). In oncology, laser microdissection was also applied to studies on the loss of heterozygosity (LOH) in tumor progression. Analysis revealed allelic loss in stromal cells resides adjacent to the cancerous cells, suggesting a contribution of stromal cells to cancer progression (Tuhkanen et al., 2004). Such an insight was possible only when tumor cells and surrounding cells were microscopically separated and analyzed by laser microdissection. Mutation analyses have also been performed with samples isolated by laser microdissection (Keohavong et al., 2004).

Laser microdissection to isolate and characterize single cells

One of the ultimate goals of a genome-wide approach such as microarray or protein array is the characterization of a single cell. Although RNA amplification from a single cell is challenging, this concept is important in neurobiology because individual neurons can play distinct roles in the maintenance of a neuronal circuit. Several groups have reported attempts at single-cell profiling in tumor cells or neural cells (Todd and Margolin, 2002; Kamme et al., 2003).

Laser microdissection for isolation of living cells

Laser microdissection is usually reserved for fixed cells. However, laser microdissection can also be used to isolate living cells from cultured cell lines (Stich et al., 2003). The behavior of isolated cells in culture can be determined from morphological or proliferative characteristics. The application of this method to tissue sections will be addressed in future studies.

Concluding remarks

Since its introduction in the late 1990s, laser microdissection has been recognized as an ideal method for preparation of homogeneous cell populations, which is invaluable for downstream analyses such as genomics or proteomics. However, no single method can be applied to all of the analyses, including previous applications of laser microdissection. Thorough understanding of the advantages and disadvantages (labor-intensive, need for RNA amplification) of the method is important for the selection of a microdissection approach, whole-tissue approach, or a combination of both approaches.

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