Technical Advance

Frozen Tumor Tissue Microarray Technology for Analysis of Tumor RNA, DNA, and Proteins

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Tissue microarray technology is a new method used to analyze several hundred tumor samples on a single slide allowing high throughput analysis of genes and proteins on a large cohort. The original methodology involves coring tissues from paraffin-embedded tissue donor blocks and placing them into a single paraffin block. One difficulty with paraffin-embedded tissue relates to antigenic changes in proteins and mRNA degradation induced by the fixation and embedding process. We have modified this technology by using frozen tissues embedded in OCT compound as donor samples and arraying the specimens into a recipient OCT block. Tumor tissue is not fixed before embedding, and sections from the array are evaluated without fixation or postfixing according to the appropriate methodology used to analyze a specific gene at the DNA, RNA, and/or protein levels. While paraffin tissue arrays can be problematic for immunohistochemistry and for RNA *in situ* hybridization analyses, this method allows optimal evaluation by each technique and uniform fixation across the array panel. We show OCT arrays work well for DNA, RNA, and protein analyses, and may have significant advantages over the original technology for the assessment of some genes and proteins by improving both qualitative and quantitative results. (*Am J Pathol* 2001, 159:1645–1650)

Recently developed high density tissue microarray technology involves arraying up to 1000 cylindrical tissue cores from individual tumors on a tissue microarray.¹ More than 200 serial sections can then be made from an individual microarray block and used for analysis of DNA, RNA, and/or proteins on a single glass slide. The technology is useful in that it allows rapid analysis of a large number of samples so that the statistical relevance of new markers can be determined in a single experiment.

In addition, altered expression levels can be correlated to amplification or deletion events in specific tumor samples using serial sections, allowing simultaneous determination of gene copy number and expression analysis of candidate pathogenic genes and suppressor genes. Arrays have been made containing numerous tumor types² as well as multiple stages and grades within individual tumor types.^{3–5} This new technology has already proven useful for rapidly characterizing the prevalence and prognostic significance of differentially expressed genes identified using cDNA array technology^{5–7} as well as genes involved in cancer development and progression.^{4,5} Tissue microarrays have also been useful in identifying genes that are targets of chromosomal amplification^{8,9} as well as to study the expression patterns of putative tumor suppressor genes.¹⁰

Some technical problems exist with the current methodology, however, relating to the fact that the arrayed samples have been pre-fixed and embedded in paraffin. The quality of the studies performed on sections from tissue array technology may be limited by the fixation methods used on the original sample. Buffered formalin solutions (and related compounds) are among the most widely used tissue fixatives. These chemicals fix the tissue by acting as progressive cross linkers between proteins and nucleic acids, by introducing modifications in RNA (adding mono-methyl groups to its bases), and by producing coordinate bonds for calcium ions; these processes can damage RNA and alter target antigenic structure by blocking or damaging antibody binding sites.^{11,12} Formalin fixation-induced alterations can make *in situ* analysis of DNA, RNA, and proteins suboptimal and variations in the duration of fixation can effect the quality and reproducibility of results.^{1,12,13} Fixation problems for FISH can be overcome by uniformly pre-fixing tissues in cold ethanol and embedding in paraffin,¹ but this approach may not be optimal for array analysis of some proteins or for RNA using *in situ* hybridization. Paraffin

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Figure 1. Frozen microarray method and HE staining. **A:** A total of 96 1.0-mm samples from solid tumor mouse xenografts (derived from Calu-6, a human lung cancer cell line) spaced 1.0 mm apart are embedded in an OCT block mounted on a plastic cassette as described in Materials and Methods. **B:** After the array is completed, a cylinder is mounted with OCT to the back of the array which readily fits into the Hacker OTF cryostat for sectioning. **C:** A 4- μ m section of the block shown in A is HE-stained to show overall integrity and spacing. **D:** 4 $\times$ magnification of the same section shows level of tissue and cell morphology maintained in the OCT array.

embedding of ethanol-fixed tissue does not prevent RNA degradation.¹⁴ In addition, while ethanol fixation of tissue and subsequent paraffin embedding circumvents formalin fixation-related problems introduced by cross-linking, there are still problems relating to the embedding, and/or deparaffinization processes such as temperature-induced antigenic alterations introduced during the embedding process.^{12,15,16} One way to avoid these problems and ensure optimal preservation of antigens and nucleic acids is to use non-fixed (fresh frozen) tissue frozen at -70°C .^{17,18} In the current study we employ a change in the methodology that circumvents some of the problems with paraffin arrays and demonstrate that frozen tissue is suitable for creating tumor tissue microarrays.

Materials and Methods

Array Construction

A human lung cancer cell line Calu-6 grown as a mouse xenograft and human breast cancer cells MDA-MB-231 grown *in vitro* and pelleted were frozen and embedded in OCT compound embedding medium (Miles, Inc. Diagnostic Division, Elkhart, IN) to test whether arrays could be cored and collected in this medium. Methodology was as published previously,¹ except that tissue biopsies (diameter 0.6 and 1.0 mm; height 3–4 mm) were punched from tumors in OCT and placed directly into an OCT array

block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD).

The recipient OCT array block was made by filling a Tissue-Tek standard cryomold (Miles, Inc.) with OCT and mounting the OCT filled mold to the base of a plastic biopsy cassette (Simport Histosette II Biopsy Cassette from Fisher Scientific with lid removed); see Figure 1A. The recipient OCT block has the same size base as the paraffin recipient block that the tissue microarrayer was made to accommodate, and therefore it was easily mounted in the tissue microarrayer (Beecher Instruments). The recipient block must be surrounded with dry ice to prevent melting. The same needle (0.6 or 1.0 mm, Beecher Instruments) was used for both coring the recipient array block and collecting the core biopsy rather than switching to a larger needle for the biopsies. The tissue in the needle was kept frozen by holding the needle against a piece of dry ice before and after punching the tissue and while dispensing the tissue core into the recipient block. Punching and coring were done slowly with minimal pressure to prevent needle breakage. The recipient array was kept frozen by placing a piece of dry ice on its upper surface at all times except when punching and filling holes. A space of one millimeter was left between each 0.6- or 1.0-mm punch.

For testing the feasibility of this method we created two arrays. One array contained 40 samples (0.6 mm in diameter). This array consisted of 20 samples of a cell line

(MDA-MB-231) frozen in OCT (frozen cells quick-thawed and pipetted into a hole in the recipient OCT block also work), and 20 biopsies of a solid tumor frozen in OCT (Calu6 mouse xenografts) cored and placed in the recipient OCT block. The second array contained 96 biopsies (1.0 mm in diameter) of solid tumors frozen in OCT (Calu6 mouse xenografts), shown in Figure 1. After the frozen tissue arrays were completed, a mounting cylinder (Hacker Instruments Inc., Fairfield, NJ) was fixed with OCT medium to the back of the array (Figure 1, A and B). 4- to 10- μ m sections of the whole block were cut from the array block using a cryostat microtome (Hacker Instruments, Inc.) and the Basic CryoJane Tape Transfer System and slides (Instrumedics Inc., Hackensack NJ). The remaining tissue array was stored at -70°C . Slides were HE-stained to assess the morphological integrity of the tissue microarray (Figure 1, C and D).

Nonradioactive RNA *in Situ* Hybridization

Nonradioactive RNA *in situ* hybridization was performed as published previously for frozen sections.¹⁹ Briefly, tissue array sections used for RNA *in situ* hybridization were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for either 10 minutes, 2 hours, or overnight at 4°C . Slides were rinsed three times in PBS for 5 minutes each and drained. Sections were covered with a prehybridization buffer (50% deionized formamide, 5X SSC, 5X Denhardt's, 750 $\mu\text{g}/\text{ml}$ torula RNA) and placed in a humid chamber at room temperature for 2 hours. Hybridization was performed by adding 0.5 μg of digoxigenin-labeled actin RNA probe (Boehringer Mannheim, Indianapolis, IN) to 10 ml of prehybridization solution in a 5-slide mailer. Tissue nucleic acid was denatured at 85°C for 10 minutes and cooled on ice. The RNA probe was omitted as a negative control to determine background due to detection reagents. Slides were hybridized overnight at 70°C , then washed in 5X SSC at 70°C for 5 minutes and in 0.2X SSC at 70°C for 60 minutes. Sections were next washed for 5 minutes in buffer B1 (0.1 mol/L maleic acid, 0.15 mol/L NaCl) and placed in a humid chamber with blocking solution (1% blocking agent, Boehringer-Mannheim) for 1 hour at room temperature. Slides were then drained and incubated at room temperature for 1 hour with a 1:2000 dilution of AP-conjugated α -digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) in B2. The antibody was drained and slides were rinsed in B1 twice for 20 minutes each. Slides were next washed in B3 (100 mmol/L Tris pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl_2) for 5 minutes. Slides were then drained but not dried and covered with BM Purple substrate (Boehringer Mannheim) overnight. Signal was postfixed in 4% paraformaldehyde in PBS, and the signal visualized using standard light microscopy.

Fluorescent *in Situ* Hybridization

Frozen tissue microarray sections were fixed in Carnoy's fixative or 95% ethanol for 10 minutes. Slides were pretreated in 2X SSC at 37°C for 30 minutes, dehydrated,

denatured in 70% formamide/2X SSC for 5 minutes at 72°C , and dehydrated again. Slides were then treated either with or without 0.4 $\mu\text{g}/\text{ml}$ proteinase K (Sigma, St. Louis, MO) at 37°C for 30 minutes. A spectrum orange chromosome 8 probe (Vysis Inc., Downer's Grove, IL) was prepared according to the manufacturer's instructions, denatured for 7 minutes at 72°C , and hybridized to the array slides overnight at 37°C in a humid chamber. Slides were washed (50% formamide/2X SSC 44°C , 15 minutes; 2X SSC, 8 minutes) and counterstained with DAPI (Vysis). Slides were visualized using standard fluorescent microscopy and photographed with Ektachrome 400 ASA slide film (Eastman Kodak, Rochester, NY).

Immunohistochemistry

Array slides for immunohistochemistry were prepared by sectioning of the block as described above, then fixed in cold 100% methanol for 15 minutes. Sections were rinsed in PBS, quenched in 0.45% hydrogen peroxide in PBS for 15 minutes, and rinsed again. Immunohistochemistry was performed using standard procedures (ABC-Elite, Vector Laboratories, Burlingame, CA). Briefly, slides were preincubated with normal goat serum and blocking avidin for 20 minutes then rinsed in PBS. Monoclonal antibodies were used for detection of α -heregulin (Santa Cruz Biotechnology, Santa Cruz, CA), and the EGF receptor (BD Biosciences, San Diego, CA) at a 1:100 dilution. Slides were incubated with the heregulin antibody and biotin in normal goat serum or with the EGF receptor antibody and biotin in normal horse serum for 1 hour and rinsed in PBS. The primary antibodies were not included in negative control experiments. Slides were incubated with the secondary antibody (biotinylated anti-rabbit IgG made in goat diluted 1:350 in normal goat serum or biotinylated anti-mouse IgG made in horse diluted 1:50 in normal horse serum, Vector Laboratories) for 1 hour. Solutions A and B (ABC-Elite) were added simultaneously for 30 minutes. Diaminobenzidine was used as a chromogen and arrays were visualized and photographed using standard light microscopy.

Results and Discussion

Microarray technology is currently a critical new technology which allows for rapid analysis of 100s to 1000s of genes, proteins, and tissue samples in expedited experimental approaches.¹ This relatively new tumor tissue technology has already shown potential in rapidly identifying and characterizing genes and markers involved in the pathogenesis of human cancers.²⁻¹⁰ To date, human malignant tissue microarrays are most commonly constructed from archival paraffin tissue blocks. The paraffin-based technology may not be optimal for studying RNA, DNA, and proteins simultaneously on a single array because FISH, RNA *in situ*, and immunohistochemistry all have different optimal fixation conditions. To address this problem, we created two test arrays (40 $\times$ 0.6 mm diameter samples and 96 $\times$ 1.0 mm samples) using a new method. This study was performed to determine whether

Figure 2. Non-radioactive RNA *in situ* hybridization. Non-radioactive RNA *in situ* hybridization with digoxigenin-labeled actin on frozen tissue microarray Calu-6 mouse xenograft sample at **A:** 20× magnification shows mRNA expression levels can be assessed using this technology. **B:** Negative control on a consecutive 4-μm section at 20× magnification shows no signal.

samples could be cored from frozen tissue samples (and cell lines) embedded in OCT compound (or directly thawed for cell lines) and placed into a frozen OCT recipient array block for sectioning and subsequent storage. The standard 0.6-mm microarray needles (Beecher Instruments), which are used for the paraffin-based microarrays, can core frozen tissue, but minimal pressure must be applied to prevent needle breakage. In our experience, larger (1.0-mm) needles (Beecher Instruments) are far sturdier. Frozen tumor tissue and cell lines embedded in OCT compound were successfully cored and placed into an OCT compound recipient block. The array was constructed with ≥ 1 mm space between punches. Arraying samples more proximally tended to cause cracking in the recipient array block. The frozen tissue array samples maintained adequate morphology as seen when sections as thin as 4 μm cut and HE stained (Figure 1, C and D). The tape transfer system (Instrumedics, Inc.) was critical to maintaining the integrity of the samples (compare array in Figure 1A to HE-stained slide made from section of 1A, shown in 1C). We obtained similar results using the tape transfer system to section a human breast tumor array (not shown). The morphology and integrity of the human breast tumor array was comparable to the array shown in Figure 1, C and D suggesting this technique should be similarly successful using fatty tissues that are generally difficult to section using standard methods (cryosectioning without the tape system).

To determine whether the tumor tissue microarray could be used for analysis of RNA, we performed non-radioactive RNA *in situ* hybridization to the tissue microarray slide using a digoxigenin-labeled actin RNA probe. Array slides were fixed for 10 minutes, 2 hours, or overnight in 4% paraformaldehyde to test whether shorter fixation times could be used for non-radioactive RNA *in situ* hybridization. Using actin as a probe, the studies demonstrated excellent preservation of intact RNA when the array section was fixed overnight in 4% paraformal-

dehyde (Figure 2). Slides fixed for 10 minutes and 2 hours showed no signal suggesting shorter fixation times may result in ineffective fixation.

The frozen tissue array should also be an excellent approach for FISH-based experiments to analyze DNA (Figure 3). To pursue this, FISH of a chromosome 8 centromere probe to the frozen tissue microarray was assayed to test whether the array could be used for *in situ* analysis of tumor DNAs. To determine which fixative works best for FISH to the frozen tissue microarray, we fixed array slides in either Carnoy's fixative or ethanol. We saw a slightly stronger signal when slides for FISH were pre-fixed with Carnoy's fixative as compared to ethanol fixation, but both worked well. We also pretreated array slides with and without proteinase K to determine whether proteinase K treatment would have an effect on the quality of hybridization and signal intensity. The proteinase K

Figure 3. Fluorescent *in situ* hybridization (FISH). FISH on Calu-6 mouse xenograft tissue microarray 4-μm section shown in Figure 1 shows intense signals with a chromosome 8 centromere probe (Vysis). Signals are easily detected on DAPI-counterstained nuclei at 100× magnification with a triple-pass filter on a fluorescent microscope.

Figure 4. Immunohistochemistry. Antibody staining for the EGF receptor on frozen array sample MDA-MB-231 (human breast cancer cell line known to express EGF receptor) shows at 4 $\times$ magnification (**A**) that staining is relatively uniform and specific across the sample and at 40 $\times$ magnification (**B**) shows expected membrane-specific staining when compared to no background staining (**C**) on serial section with secondary antibody staining only, and HE staining (**D**) of the same sample from a serial section of the array.

treatment did not improve hybridization efficiency or signal intensity, as the probe penetrated the frozen tissue equally well under both conditions.

Finally, we performed immunohistochemistry on the tumor tissue microarray with antibodies for the EGF receptor (HER-1) and heregulin. The EGF receptor staining is uniform across the sample (Figure 4A) and gives the expected membrane-associated staining, as seen when comparing the EGF receptor-stained sample with a serial HE-stained section (Figure 4, B and D). There is no background staining using the secondary antibody alone (Figure 4C). Similar results were obtained using the heregulin antibody except the staining showed the expected diffuse cytoplasmic signal (not shown). Antibodies to heregulin and the EGF receptor both result in cell-specific signals showing this methodology will be useful for immunohistochemical based protein analyses as well.

In summary, frozen tissue microarrays appear to provide excellent target material for the study of DNA, RNA, and proteins by fixing each array slide in a manner specific to the corresponding technique used. The disadvantage of the frozen tissue microarray technology is that there is some distortion of cell morphology and tissue architecture compared to formalin fixed paraffin-embedded arrays. This is commonly seen when comparing frozen sections to paraffin sections. Another drawback to the frozen tissue microarray technology is that less samples can be embedded on a single array because the OCT compound may bend and crack when samples are placed at less than 1 mm apart. In our test array, we easily fit 96 samples (with 1 mm diameter) in the array block with room to add additional samples if needed (Figure 1A). This sample size seems to be within the range of what is commercially acceptable for paraffin

arrays. For example, currently paraffin arrays containing up to sixty individual tissue samples (with 2 mm diameter) and up to 200 individual tissue samples (with 0.6 mm diameter) can be purchased from SuperBioChips Laboratories, Seoul, Korea, and Invitrogen Corp., San Diego, CA, respectively. To fit more samples on the frozen array, a larger plastic cryomold can be used, or alternatively a smaller coring needle can be used to fit more samples in the same space. The smaller needle biopsies have two disadvantages, the smaller needles break more easily and there is less representation of the tumor the biopsy is derived from. One way to get around the needle breakage problem is to let the frozen tissue thaw a little before taking a biopsy of the sample. In our experience, briefly thawing frozen tissue did not effect actin RNA quality, but it may have an effect on less abundant, less stable messages.

To improve the problem of sampling error, several biopsies from each sample can be taken. A recent study of immunohistochemistry on paraffin arrays has shown that double sampling of 0.6 mm diameter punches of tumors leads to representation of the original tumor in at least 95% of the tumors on the array.²⁰ Double punching may also improve representation using the larger needles as well. Separate recipient arrays can be made representing duplicate samples so that total sample number doesn't have to be limited by double-punching.

The advantage of the frozen microarray approach, stems from the fact that certain antibodies, DNA, and RNA probes do not perform optimally in pre-fixed paraffin-embedded tissues. These reagents are likely to work very well using the technology presented here. Another advantage of the frozen tissue microarrays is that those procedures requiring fixation can be conducted in samples fixed in an identical manner. Therefore, a higher

proportion of the arrayed samples may be included in the final analysis than with the paraffin-embedded tumor microarrays. Frozen tumor tissue microarrays provide an excellent way to store and analyze tumor samples and may prove useful for identifying novel molecular targets for diagnosis, prognosis, and therapy of cancer, as well as for validation of cDNA microarray studies. By allowing simultaneous analysis of uniformly and optimally fixed DNA, RNA, and proteins from 100s of tumor samples, this technology may lead to advances in the understanding of tumor pathobiology.

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References

- Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998, 4:844–847
- Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, Mihatsch MJ, Kallioniemi OP, Sauter G: Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* 1999, 5:1966–1975
- Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser T, Mihatsch MJ, Kallioniemi OP, Sauter G: High-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarray screening in renal cell carcinoma. *Am J Pathol* 1999, 154:981–986
- Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP: Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence *in situ* hybridization on tissue microarrays. *Cancer Res* 1999 59:803–806
- Bubendorf L, Kolmer M, Kononen J, Koivisto P, Mousses S, Chen Y, Mahlamaki E, Schraml P, Moch H, Willi N, Elkahoul AG, Pretlow TG, Gasser TC, Mihatsch MJ, Sauter G, Kallioniemi OP: Hormone therapy failure in human prostate cancer: analysis by complementary DNA and tissue microarrays. *J Natl Cancer Inst* 1999, 91:1758–1764
- Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser T, Mihatsch MJ, Kallioniemi OP, Sauter G: Identification of prognostic parameters for renal cell carcinoma by cDNA arrays and cell chips. *Verh Dtsch Ges Pathol* 1999, 83:225–232 (in German)
- Barlund M, Forozan F, Kononen J, Bubendorf L, Chen Y, Bittner ML, Torhorst J, Haas P, Bucher C, Sauter G, Kallioniemi OP, Kallioniemi A: Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. *J Natl Cancer Inst* 2000, 92:1252–1259
- Barlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, Kallioniemi OP, Kallioniemi A: Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. *Cancer Res* 2000, 60:5340–5344
- Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Kronagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schonenberger A, Flury R, Jager P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G: High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* 2000, 157:787–794
- Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC, Koivisto P, Lack EE, Kononen J, Kallioniemi OP, Gelmann EP: Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. *Cancer Res* 2000, 60:6111–6115
- Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K: Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res* 1999, 27:4436–4443
- Werner M, Chott A, Fabiano A, Battifora H: Effect of formalin tissue fixation and processing on immunohistochemistry. *Am J Surg Pathol* 2000, 24:1016–1019
- Specht K, Richter T, Muller U, Walch A, Werner M, Hoffer H: Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* 2001, 158: 419–429
- Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR: Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinog* 1999, 25:86–91
- Battifora H, Kopinski M: The influence of protease digestion and duration of fixation on the immunostaining of keratins: a comparison of formalin and ethanol fixation. *J Histochem Cytochem* 1986, 34: 1095–1100
- Penault-Llorca F, Adelaide J, Houvenaeghel G, Hassoun J, Birnbaum D, Jacquemier J: Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. *J Pathol* 1994, 173:65–75
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, 244:707–712
- Battifora H: Effect of fixatives and fixation times on tissues. *Am J Clin Pathol* 1991, 96:144–145
- Hogan BLM, Beddington R, Costantini F, Lacy E: Manipulating the Mouse Embryo: A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press, 1994
- Camp RL, Charette LA, Rimm DL: Validation of tissue microarray technology in breast carcinoma. *Lab Invest* 2000, 80:1943–1949