Breast Cancer Online
www.bco.org

Breast Cancer Online (www.bco.org) 2005; **8**(7),
doi:10.1017/S1470903105003111

ISSN 1470-9031

Focus On

Tissue microarrays – automated analysis and future directions

K. A. DiVito, R. L. Camp

Department of Pathology and the Yale Cancer Center, Yale University, School of Medicine, New Haven, CT, USA.

Abstract Tissue microarrays have rapidly become a vital tool in the analysis of protein biomarkers on large patient cohorts. Composed of hundreds of miniature histologic tissue cores taken from patient tumors and arrayed in a grid pattern, tissue microarrays have great potential for automation. However, unlike DNA/RNA expression arrays, they contain spatial information, such that successful automation requires both the ability to quantitate *and* localize biomarkers within the tissue. This review traces tissue microarray analysis from the simplest of systems (visual inspection) to dedicated, automated quantitative analyzers capable of localizing biomarkers to sub-cellular compartments. It focuses on the importance of automating the process of tissue microarray analysis, while commenting on the inherent problems associated with both manual and automated analysis. Finally, it discusses the future of automated tissue microarray analysis, emphasizing the many areas of development and potential improvement.

Keywords: Automated analysis; Immunofluorescence; Immunohistochemistry; Tissue microarrays

The quantitation of biomarkers on tissue microarrays (TMAs) provides numerous technical challenges for the development of automated systems capable of reading microarrays and translating image information into useable data. Unlike DNA/RNA expression arrays, each spot (or histospot) on a TMA represents a miniature histologic section of tissue that contains complex spatial information that can dramatically affect the quantitative analysis of biomarkers [1–3]. The location and expression level of biomarkers on TMAs is generally determined using target-specific antibodies tagged with fluorescent dyes or enzymes that deposit chromogenic and/or fluorescent substrates. In some cases a given biomarker may only be represented in a subset of cells within a TMA spot (e.g. tumor vs. stromal cells) or may be found differentially expressed in different sub-cellular compartments within cells (e.g. membrane vs. cytoplasm vs. nucleus). In some cases, the cell of interest may only represent a small percentage of the analyzed area, such that an analysis of biomarker intensity within the entire area is meaningless. Accurate quantification, therefore, requires accurate measurement of both biomarker expression and location. Indeed, the importance of preserving the spatial information has proven to be an essential component of automated TMA analysis and is incorporated, in some degree, into most systems currently available for automated TMA analysis.

Correspondence to: Robert L. Camp, Department of Pathology, Yale University, School of Medicine, 310 Cedar Street, P.O. Box 208023, New Haven, CT 06520-8023, USA. E-mail: robert.camp@yale.edu; Tel: +1 203 785 6340

Publication date 29/07/05
BCO/311/2004/FO

Quantitative visual analysis of tissue microarrays

The simplest method of biomarker quantitation relies on manual (visual) inspection, usually by a pathologist or someone trained in basic histology, to determine accurate expression levels in histologically complex tissues. In general, expression of biomarkers is scored

Table 1. Commercially available tissue microarray analyzers.

TMA system	Licensed by/website
BLISS slide scanning workstation	Bacus Laboratories, Inc., Lombard, IL http://www.bacuslabs.com
Automated Cellular Imaging System (ACIS®)	Clarient, Inc (formerly ChromaVision, Inc.), San Juan Capistrano, CA http://www.chromavision.com
ScanScope® Virtual microscopy solutions	Aperio Technologies, Inc., Vista, CA http://www.aperio.com/
TissueAnalytics™	Icoria™ Research Triangle Park, NC http://www.icoria.com/
GenoMX™ Vision	Biogenex San Ramone, CA http://www.biogenex.com/
Discovery TM™	Molecular Devices, Corp., Sunnyvale, CA http://www.moleculardevices.com/
Automated Quantitative Analysis (AQUA)	HistoRx, New Haven, CT http://www.historx.com/
Pathological Image Analysis and Management (PATHIAM)	Bioimagene, Inc., San Jose, CA http://www.bioimagene.com/
Ariol® Pathology Workstation	Applied Imaging Corp., San Jose, CA http://www.aicorp.com

on an ordinal (0, 1+, 2+, 3+) scale, which in some cases is combined with a scored interpretation of the markers' overall distribution [4,5]. Manual scoring has been successfully used in hundreds of manuscripts on TMAs with a variety of tissue types. Of course, there are limitations to visual inspection of microarrays. At best, manual inspection is only semi-quantitative, reducing biomarker expression – which generally occurs in nature as a continuous, normal distribution – to an ordinal scale. Visual inspection can also be confounded by the inherently subjective nature of human observation, affected by context (e.g. the amount of tumor present, background staining, stromal staining, and even the order in which histospots are observed) [6]. These issues can lead to low inter- and intra-observer variability [7,8]. In some cases, they can preclude the discovery of subtle sub-populations that cannot be identified using manual analysis [9,10].

Current methods of automated tissue microarray analysis

The potential benefits of automated analysis were realized early on as a way to promote TMAs as a method for high-throughput biomarker discovery [11]. Indeed, perhaps the greatest argument against manual scoring, aside from its inherent subjectivity, is the time consuming and tedious burden of repetitively scoring hundreds to thousands of histospots. More recently, systems for the automated reading and image archiving have eliminated some of the most tedious aspects of TMA analysis and permit simultaneous, web based, visual inspection of individual histospots at multiple institutions [12–15]. Several researchers have developed TMA analysis systems out of existing technologies and software [16–19]. These systems dovetail into the recent development of commercially available, dedicated TMA readers and software packages (Table 1), which provide automated quantitation of biomarkers in addition to image acquisition [1]. Unfortunately, the cost of these systems (usually greater than $200 000) is prohibitive for many researchers. Most of these systems utilize chromogenic (i.e. 'brown-stain') substrates to quantify biomarkers. Localization of biomarkers is achieved by counterstaining the TMA with traditional histochemical dyes (e.g. hematoxylin, eosin). Automated systems then use morphometric analyses based on size, shape, and color to distinguish tumor from stroma, nuclei from cytoplasm, etc. This technology has achieved higher accuracy than manual scoring for a number of biomarkers including Her-2/neu, ER, and p21 [20–24]; although its use in tissue microarrays *per se* is in its infancy [25,26]. However, since chromogenic substrates rely on opacity (i.e. optical density) to assess intensity, there is a finite limit to detection (namely 100% opacity) as governed by Beer's law [27]. As optical density is a logarithmic rather than linear representation of the amount of light absorbed by a chromophore, large changes in protein concentration can affect only small changes in optical density. This is particularly true in the range of optical densities used in immunohistochemistry (OD: 0.5–2.0) [28], thus complicating quantitation using chromophores.

An alternative approach has been the development of systems for immunofluorescence-based antigen detection [10,29]. In theory, such systems should provide quantitation that is more sensitive, easier to localize, and with a broader dynamic range. Unlike chromogenic dyes, immunofluorescence is epifluorescent rather than transmitted, so there is no theoretical limit to detection. Fluorophores come in a wide array

of colors, permitting the use of multiple fluorescently tagged antibodies on a single microarray. Rather than using morphometric analysis to localize biomarker signals, fluorescent analysis co-localizes these signals using specific tags to cells and/or sub-cellular compartments of interest. For example, epithelial tumor cells might be distinguished from surrounding stroma using a fluorescently labeled anti-cytokeratin antibody. Nuclei can be distinguished from cytoplasm using diamidino-2-phenylindole (DAPI). Multiple publications have demonstrated the utility of fluorescence-based automated TMA analysis in a variety of tumor types including breast, colon, melanoma, oropharyngeal, prostate, and carcinoid [9,10,30–35].

Of course, there are several disadvantages for fluorescent-based TMA analysis. First, epifluorescent microscope equipment is generally higher cost and more complex than traditional light microscopy. Second, although some biomarkers can be visualized with specific antibodies directly conjugated to fluorophores, most require some form of enzymatic amplification. Similar to chromogenic methods, fluorescent systems generally use peroxidase-conjugated antibodies to catalyze the deposition of tyramide-containing fluorescent substrates [36–38]. This enzymatic process, though capable of dramatic amplification of low level biomarkers (up to 100-fold [39,40]) is, like all enzymatic reactions (e.g. polymerase chain reaction (PCR)), inherently non-linear particularly at high expression levels. Third, formalin fixed tissues exhibit strong background autofluorescence in the wavelengths of the most common fluorophores (e.g. fluresein isothiocyanate (FITC), rhodamine, phycoerythrin). Fortunately, tissue autofluorescence diminishes markedly in the far red to infrared spectrum, and the development of dyes (e.g. Cy5, Cy7, Alexa-647, and Alexa-750) and digital cameras capable of visualizing signals into the near-infrared have minimized this issue. Fluorescence-based systems have the added potential to multiplex TMA reading (i.e. the simultaneous staining and analysis of multiple biomarkers each tagged with a different fluorophore, on a single TMA). The flexibility of fluorescence-based systems to co-localize targets tagged with different fluorophores, permits the quantification and localization of biomarkers in a wide array of fluorescently tagged sub-cellular compartments (e.g. DAPI-stained nuclei, antibody tagged membranes, mitochondria, vessicles, or golgi). This method has proven beneficial in the study of several biomarkers (e.g. beta-catenin in colon carcinoma, AP-2 in melanoma) that exhibit sub-cellular compartmentalization [10]. Furthermore, it provides the potential to develop 'virtual' compartments. Thus, a researcher might define a compartment as a particular growth factor receptor (e.g. using a receptor-specific antibody) and then analyze the amount of phosphorylated receptor within that compartment (using a phospho-specific antibody).

The future of automated tissue microarray analysis

The ultimate (and highly demanding) goal of automated TMA analysis is to provide a standard, reproducible, efficient, sensitive, and specific method of biomarker quantitation. Such a system would ultimately report biomarker expression as molecules per-cell or unit-area. However the attainment of this goal will necessarily include advancements in every aspect of tissue microarray production, staining, reading and analysis. New and better methods for tissue preservation (in contrast to formalin fixation) may do a better job of preserving antigenicity and eliminating the need for antigen retrieval [41]. Newer fluorophores and non-enzymatic amplification procedures may provide linear signals across an even broader dynamic range, potentially permitting the detection of single molecules. In particular, quantum dots have shown some promise in this regard [42]; although their successful use on TMAs is, as yet, unproven. The development of fluorophores and cameras capable of imaging even farther into the infrared spectrum (outside of tissue autofluorescence) may facilitate the study of multiple biomarkers on a single array. Also important is the development of quality control standards consisting of specific analyte molecules at set concentrations. These can ultimately be incorporated into microarrays as a series of cell lines or spots containing recombinant protein to provide a dilution curve from which specific biomarker concentrations can be determined [1]. Perhaps the most vital component of automated TMA analysis is the development of databases and biostatistical tools to rigorously analyze the millions of biomarker assays likely to come from high-throughput analysis. Fortunately, dedicated methods for cutpoint analysis [43] and hierarchical clustering of TMAs [44–51], akin to those created for RNA/DNA expression arrays, are currently being developed.

Acknowledgements

Supported by grants from the Breast Cancer Alliance and the NIH (K08 ES11571).

References

1. Giltnane JM, Rimm DL. Technology insight: identification of biomarkers with tissue microarray technology. *Nat Clin Pract Oncol* 2004; **1**: 104–111.

2. Simon R, Mirlacher M, Sauter G. Tissue microarrays. *Biotechniques* 2004; **36**: 98–105.
3. Kononen J, Bubendorf L, Kallioniemi A, *et al*. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844–847.
4. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 1999; **17**: 1474–1481.
5. McCarty Jr KS, Szabo E, Flowers JL, *et al*. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res* 1986; **46**: 4244s–4248s.
6. Anderson BL, Winawer J. Image segmentation and lightness perception. *Nature* 2005; **434**: 79–83.
7. Thomson TA, Hayes MM, Spinelli JJ, *et al*. HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent *in situ* hybridization. *Mod Pathol* 2001; **14**: 1079–1086.
8. Kay EW, Walsh CJ, Cassidy M, *et al*. C-erbB-2 immunostaining: problems with interpretation. *J Clin Pathol* 1994; **47**: 816–822.
9. Camp RL, Dolled-Filhart M, King BL, Rimm, DL. Quantitative analysis of breast cancer tissue microarrays shows that both high and normal levels of HER2 expression are associated with poor outcome. *Cancer Res* 2003; **63**: 1445–1448.
10. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002; **8**: 1323–1327.
11. Theillet C. Full speed ahead for tumor screening. *Nat Med* 1998; **4**: 767–768.
12. Bova GS, Parmigiani G, Epstein JI, *et al*. Web-based tissue microarray image data analysis: initial validation testing through prostate cancer Gleason grading. *Hum Pathol* 2001; **32**: 417–427.
13. Manley S, Mucci NR, De Marzo AM, Rubin MA. Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome: the prostate specialized program of research excellence model. *Am J Pathol* 2001; **159**: 837–843.
14. Chen W, Foran DJ, Reiss M. Unsupervised imaging, registration and archiving of tissue microarrays. *Proc AMIA Symp* 2002; 136–139.
15. Chen W, Reiss M, Foran DJ. A prototype for unsupervised analysis of tissue microarrays for cancer research and diagnostics. *IEEE Trans Inf Technol Biomed* 2004; **8**: 89–96.
16. Liu CL, Prapong W, Natkunam Y, *et al*. Software tools for high-throughput analysis and archiving of immunohistochemistry staining data obtained with tissue microarrays. *Am J Pathol* 2002; **161**: 1557–1565.
17. Vrolijk H, Sloos W, Mesker W, *et al*. Automated acquisition of stained tissue microarrays for high-throughput evaluation of molecular targets. *J Mol Diagn* 2003; **5**: 160–167.
18. Matkowskyj KA, Schonfeld D, Benya RV. Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software photoshop and matlab. *J Histochem Cytochem* 2000; **48**: 303–312.
19. Matkowskyj KA, Cox R, Jensen RT, Benya RV. Quantitative immunohistochemistry by measuring cumulative signal strength accurately measures receptor number. *J Histochem Cytochem* 2003; **51**: 205–214.
20. Wang S, Saboorian MH, Frenkel EP, *et al*. Assessment of HER-2/neu status in breast cancer. Automated cellular imaging system (ACIS)-assisted quantitation of immunohistochemical assay achieves high accuracy in comparison with fluorescence *in situ* hybridization assay as the standard. *Am J Clin Pathol* 2001; **116**: 495–503.
21. Sauter G, Simon R, Hillan K. Tissue microarrays in drug discovery. *Nat Rev Drug Discov* 2003; **2**: 962–972.
22. Simon R, Sauter G. Tissue microarray (TMA) applications: implications for molecular medicine. *Expert Rev Mol Med* 2003; **2003**: 1–12.
23. Hoang MP, Sahin AA, Ordonez NG, Sneige N. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. *Am J Clin Pathol* 2000; **113**: 852–859.
24. Allred DC, Swanson PE. Testing for erbB-2 by immunohistochemistry in breast cancer. *Am J Clin Pathol* 2000; **113**: 171–175.
25. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. *Am J Surg Pathol* 2002; **26**: 312–319.
26. Faith DA, Isaacs WB, Morgan JD, *et al*. Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate* 2004; **61**: 215–227.
27. Rees VH, Laurence DJ. The correspondence with Beer's law for the optical density of stained protein patterns on filter paper as a function of surface protein concentration. *Clin Chem* 1955; **1**: 329–344.
28. Jonker A, Geerts WJ, Chieco P, *et al*. Basic strategies for valid cytometry using image analysis. *Histochem J* 1997; **29**: 347–364.
29. Jubb AM, Landon TH, Burwick J, *et al*. Quantitative analysis of colorectal tissue microarrays by immunofluorescence and *in situ* hybridization. *J Pathol* 2003; **200**: 577–588.
30. Yu Z, Weinberger PM, Provost E, *et al*. beta-Catenin functions mainly as an adhesion molecule in patients with squamous cell cancer of the head and neck. *Clin Cancer Res* 2005; **11**: 2471–2477.
31. Yu Z, Weinberger PM, Haffty BG, *et al*. Cyclin d1 is a valuable prognostic marker in oropharyngeal squamous cell carcinoma. *Clin Cancer Res* 2005; **11**: 1160–1166.
32. Kidd M, Eick G, Shapiro MD, *et al*. Microsatellite instability and gene mutations in transforming growth factor-beta type II receptor are absent in small bowel carcinoid tumors. *Cancer* 2005; **103**: 229–236.
33. Rubin MA, Zerkowski MP, Camp RL, *et al*. Quantitative determination of expression of the prostate cancer protein alpha-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. *Am J Pathol* 2004; **164**: 831–840.
34. Berger AJ, Camp RL, Divito KA, *et al*. Automated quantitative analysis of HDM2 expression in malignant melanoma shows association with early-stage disease and improved outcome. *Cancer Res* 2004; **64**: 8767–8772.
35. Divito KA, Berger AJ, Camp RL, *et al*. Automated quantitative analysis of tissue microarrays reveals an association between high Bcl-2 expression and improved outcome in melanoma. *Cancer Res* 2004; **64**: 8773–8777.
36. Bobrow MN, Litt GJ, Shaughnessy KJ, *et al*. The use of catalyzed reporter deposition as a means of signal

amplification in a variety of formats. *J Immunol Meth* 1992; **150**: 145–149.
37. van Gijlswijk RP, Zijlmans HJ, Wiegant J, *et al*. Fluorochrome-labeled tyramides: use in immunocytochemistry and fluorescence *in situ* hybridization. *J Histochem Cytochem* 1997; **45**: 375–382.
38. Adams JC. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem* 1992; **40**: 1457–1463.
39. Schmidt BF, Chao J, Zhu Z, *et al*. Signal amplification in the detection of single-copy DNA and RNA by enzyme-catalyzed deposition (CARD) of the novel fluorescent reporter substrate Cy3.29-tyramide. *J Histochem Cytochem* 1997; **45**: 365–373.
40. Hunyady B, Krempels K, Harta G, Mezey E. Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *J Histochem Cytochem* 1996; **44**: 1353–1362.
41. Gillespie JW, Best CJ, Bichsel VE, *et al*. Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am J Pathol* 2002; **160**: 449–457.
42. Wu X, Liu H, Liu J, *et al*. Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nat Biotechnol* 2003; **21**: 41–46.
43. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res* 2004; **10**: 7252–7259.
44. Alkushi A, Irving J, Hsu F, *et al*. Immunoprofile of cervical and endometrial adenocarcinomas using a tissue microarray. *Virchows Arch* 2003; **442**: 271–277.
45. Shi T, Seligson D, Belldegrun AS, *et al*. Tumor classification by tissue microarray profiling: random forest clustering applied to renal cell carcinoma. *Mod Pathol* 2005; **18**: 547–557.
46. Engellau J, Persson A, Bendahl PO, *et al*. Expression profiling using tissue microarray in 211 malignant fibrous histiocytomas confirms the prognostic value of Ki-67. *Virchows Arch* 2004; **445**: 224–230.
47. Makretsov NA, Huntsman DG, Nielsen TO, *et al*. Hierarchical clustering analysis of tissue microarray immunostaining data identifies prognostically significant groups of breast carcinoma. *Clin Cancer Res* 2004; **10**: 6143–6151.
48. Au NH, Cheang M, Huntsman DG, *et al*. Evaluation of immunohistochemical markers in non-small cell lung cancer by unsupervised hierarchical clustering analysis: a tissue microarray study of 284 cases and 18 markers. *J Pathol* 2004; **204**: 101–109.
49. Mao Y, Wu J, Skog S, *et al*. Expression of cell proliferating genes in patients with non-small cell lung cancer by immunohistochemistry and cDNA profiling. *Oncol Rep* 2005; **13**: 837–846.
50. Warford A, Howat W, McCafferty J. Expression profiling by high-throughput immunohistochemistry. *J Immunol Meth* 2004; **290**: 81–92.
51. Dhanasekaran SM, Barrette TR, Ghosh D, *et al*. Delineation of prognostic biomarkers in prostate cancer. *Nature* 2001; **412**: 822–826.