Layered Peptide Array for Multiplex Immunohistochemistry

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Layered peptide array is a new methodology for multiplex molecular measurements from two-dimensional life science platforms. The technology can be used in several different configurations depending on the needs of the investigator. Described here is an indirect layered peptide array (iLPA) that is capable of measuring proteins on a solid surface, such as target antigens on a tissue section. A prototype iLPA system was developed and subsequently examined for reproducibility and specificity and then compared with standard immunohistochemistry. Semiquantitative, multiplex proteomic analysis of histological sections was achieved with up to 20 membranes. The experimental variability was 18%. Overall, the data suggest that iLPA technology will be a relatively simple and inexpensive method for molecular measurements from tissue sections. (J Mol Diagn 2007, 9:297–304; DOI: 10.2353/jmoldx.2007.060143)

In today's genomic era, there is an ongoing shift of laboratory and clinical studies toward multiplex molecular measurements of samples, allowing investigators to understand biological behavior in terms of the overall regulation of the genome, transcriptome, and proteome.^{1,2} These efforts are enabled by the recent development of new technologies that simultaneously analyze a large number of molecular species. For example, expression arrays can determine gene expression levels of essentially the entire cellular complement of mRNA.³ Likewise, new proteomic technologies are facilitating global views of protein amounts and activation status.^{4,5} These high-throughput data sets are then the basis for a variety of new and innovative bioinformatics tools that provide

unique insights into biological states and disease processes.^{6,7}

However, the study of histological sections presents a number of challenges compared with other sample types. In particular, it is difficult to maintain the two-dimensional histopathological information present within a sample while also performing multiplex molecular analysis. To some degree, the development of laser-based microdissection tools has solved this problem.⁸ However, microdissection studies are tedious and time-consuming, frequently requiring the user to pool together the procured cells and thus lose important geographic information of the cell populations and subpopulations that are captured.

Thus, there is a need in the molecular pathology community for analysis methodologies that combine two-dimensional histopathology with multiplex arrays.⁹ Indirect layered peptide array (iLPA) technology is one such example. In the present study, we describe a prototype version of iLPA and assess its capability for high-throughput tissue section measurements.

Materials and Methods

Tissue Samples

Prostatectomy cases were obtained from the National Institutes of Health and the National Naval Medical Center under an Institutional Review Board-approved protocol. Whole-mount prostate cancer cases were ethanol-fixed and paraffin-embedded as described previously.¹⁰ Tissue sections were cut to 5- to 10- μ m thickness for the iLPA protocol. Labial minor salivary gland tissues were obtained from nine patients with primary Sjögren's syndrome and two healthy volunteers and were acquired and used in accordance with approvals from the National Institute of Dental and Craniofacial Research human subject review committee. Immediately after removal, specimens were placed in OCT compound (Miles, Elkhart, IN),

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Table 1. Antibodies and Antigens Used in Study

Antibody	Peptide/antigen	Catalog number	Company	Dilutions
Cytokeratin 7 goat anti-human	Cytokeratin 7 peptide	sc-17116	Santa Cruz Technology	1:10 to 1:50
Cytokine AE1/AE3 mouse anti-human		sc-17116p 08-0132	Santa Cruz Technology Zymed	2 μ g/ml Prediluted
AQP5 goat anti-human	AQP5 peptide	sc-9890	Santa Cruz Technology	1:10 to 1:50
CD4 goat anti-human		sc-9890p	Santa Cruz Technology	2 μ g/ml
	CD4 peptide	sc-1140	Santa Cruz Technology	1:10 to 1:50
Muscarinic M3-acetylcholine receptor M3 goat anti-human		sc-1140p	Santa Cruz Technology	2 μ g/ml
		sc-7474	Santa Cruz Technology	1:10 to 1:50
	Muscarinic M3-acetylcholine receptor M3 peptide	sc-7474p	Santa Cruz Technology	2 μ g/ml
Lactoperoxidase rabbit anti-bovine		RAB/LPO	Nordic Immunology, Tilburg, The Netherlands	1:50
	Lactoperoxidase antigen/biotinylated	L8257/ L4134	Sigma-Aldrich, St. Louis, MO	25 μ g/ml
Caspase 3 goat anti-human	Caspase 3 peptide	sc-1225	Santa Cruz Technology	1:10 to 1:50
PSA goat anti-human antibody		sc-1225p	Santa Cruz Technology	2 μ g/ml
		sc-7638	Santa Cruz Technology	1:10 to 1:50
	PSA peptide	sc-7638P	Santa Cruz Technology	2 μ g/ml
Glial fibrillary acidic protein mouse anti-porcine, anti-human antibody	Albumin protein	A9511	Sigma-Aldrich	30 μ g/ml
		MAB360	Chemicon International, Temecula, CA	1:10
	Glial fibrillary acidic protein, porcine	AG230	Chemicon International	1.5 μ g/ml
Dextran polymer goat anti-mouse Ig streptavidin		Code OA354 (custom-made)	DAKO	1:10; 1:100

snap-frozen in methyl butane on dry ice, held overnight at -70°C , and then stored in liquid nitrogen until use. Tissue samples were cut at a $10\text{-}\mu\text{m}$ thickness for the iLPA protocol. Each section was placed on a charged glass slide. Tissue microarray (TMA) slides were obtained from the Tissue Array Research Program (TARP) lab at the National Cancer Institute (<http://www.cancer.gov/tarp>). One section of frozen adenocarcinoma originating from colon and one frozen schwannoma were used in the study. These tissues were obtained from the University of Virginia, Charlottesville, VA, under an Institutional Review Board-approved protocol.

Layered Peptide Arrays

P-FILM Smart Antibody Affinity membranes were used in the study (commercially available, exclusively from 20/20 GeneSystems, Inc., Rockville, MD). The membranes were cut to appropriate sizes to fit the tissue section slides being analyzed and were coated with peptides/proteins.

iLPA Protocol

P-FILM membranes were coated with peptides according to Table 1. Ethanol-fixed prostate tissue or formalin-fixed TMA sections were placed in a 60°C oven for 1 hour

followed by two consecutive 5-minute incubation steps with xylenes. Rehydration with alcohols was performed using 100, 95, and 70% alcohol. Sections were heat-treated (when needed) with citrate buffer (Lab Vision, Fremont, CA) and placed in a tender cooker (NordicWare, Minneapolis, MN) for 20 minutes followed by 20 minutes of cooling. Sections were blocked for 10 minutes with blocking serum (Casein; Vector Labs, Burlingame, CA) and incubated for 1 hour at room temperature with a cocktail of the primary antibodies according to Table 1. The membrane apparatus was stacked in the following order:

1. Binding buffer (sodium phosphate buffer, pH 7; GE Healthcare, Little Chalfont, Buckinghamshire, UK)-coated control membrane.

2. Transfer buffer-coated membrane [transfer buffer: 50 ml of 1 mol/L Tris-HCl, pH 8.0 (Quality Biological Inc., Gaithersburg, MD); 30 ml of 5 mol/L NaCl (K-D Medical, Columbia, MD); 0.5 ml of 0.874 mol/L Tween 20 (Bio-Rad, Hercules, CA), and 920 ml of distilled water].

3. P-FILM peptide- or protein-coated membranes.

4. Three transfer pads (Eastman Kodak Co., Rochester, NY) soaked in transfer buffer.

The stack was placed in a plastic pocket (Kapak Corp., Minneapolis, MN), sealed, and placed on a hot plate (HOT Orbital; Armalab, Bethesda, MD) at 37°C for 1 hour and 45°C for a 2nd hour. The stack was disassembled.

bled, and the membranes were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Technology, Santa Cruz, CA). The membranes were dried and scanned with a Typhoon 9410 scanner and a 520 BP 40 fluorescence filter with PMT 600 (fluorescein isothiocyanate: absorption 490 nm, emission 520 nm) (GE Healthcare).

Frozen tissues (minor salivary gland tissues, brain tissue, and intestinal tissue) were fixed for 10 minutes in ice-cold acetone and a similar protocol for iLPA was performed with minor modifications: The incubation period in the hot orbit was shortened to 30 minutes in 37°C and 30 minutes in 45°C.

Immunohistochemistry (IHC) Protocol

Tissue sections were cut to 5- μ m thickness and stained with various antibodies according to a standard IHC protocol (DAKO EnVision⁺ system; Carpinteria, CA). In brief, IHC reactions were carried out either on nonpretreated or heat-treated sections according to the following conditions. Sections were blocked for 10 minutes with a peroxidase block (DAKO EnVision⁺ system). Sections were incubated for 1 hour at room temperature with a primary antibody according to Table 1. After a washing step with phosphate-buffered saline (Invitrogen Corp, Grand Island, NY), sections were incubated for 10 minutes with an horseradish peroxidase-labeled polymer, and a signal was subsequently detected by 3,3'-diaminobenzidine substrate chromogen, which results in a brown-colored precipitate at the antigen site (DAKO EnVision⁺ system). Slides were counterstained with hematoxylin (Zymed Laboratories, South San Francisco, CA). Negative reactions (ie, negative cells) were identified by the absence of the precipitate and a blue counterstain.

Image Analysis

All IHC images were analyzed for the total number of cells present, the number of positively stained cells, and the number of nonstained cells or for intensity of signal using the ImagePro Analysis System (ImagePro 4.5; Cybernetics, Chevy Chase, MD) as previously described.¹¹ For iLPA analysis, images of the membranes were imported to the ImagePro 4.5 analysis software, and the optical density was calculated by the program for each tissue on the membrane by marking its borders. The optical density is defined according to the following formula: $OD = -\log_{10}(x/256)$, with 256 representing the total number of gray levels in the image and x the individual level of gray of each object. The data were then imported to Microsoft Excel 2000 (Microsoft, Seattle, WA) and saved as a spreadsheet. Enhancement of images was performed in the ImagePro 4.5 program using the automatic feature "best-fit" and is presented as an "enhanced" image.

The data from Microsoft Excel were imported to Partek-Pro 6.2. (Partek Inc., St. Charles, MO). Principal component analysis, group profile, analysis of variance statistics, and SD modules of the PartekPro software package were used to analyze the results.

Figure 1. Schematic of the iLPA system. Each membrane is coated with a different peptide or antigen specific for an antibody of interest. An antibody set is applied to a tissue section and subsequently captured and analyzed by the appropriate membrane, while maintaining the two-dimensional information present in the sample.

Results and Discussion

The iLPA platform is shown schematically in Figure 1. Membranes were coated with peptides specific to antigens of interest, and tissue sections were then incubated with a cocktail of antibodies against target proteins. After washing, the antibodies were released from the section and passed through the analysis layers while maintaining their two-dimensional position. The antibodies were specifically captured by their target peptides and subsequently detected using standard secondary antibody-based methods.

The protocol for releasing the antibody from the tissue section was determined empirically, starting with traditional column-based methods for antibody purification. The effect of different buffers, salt concentrations, and pH conditions on both the antibody detachment step and the subsequent antibody re-binding step to the membrane was determined. Ultimately, release and re-binding of antibodies was optimal using sodium phosphate buffer, pH 7, and subjecting the histology slide and membranes to heat and capillary fluid flow as described in Materials and Methods. This protocol effectively released the majority of bound antibody from the tissue section and permitted efficient re-binding of the antibody to its appropriate peptide-coated membrane.

Figure 2 demonstrates analysis of prostate-specific antigen (PSA) in a whole-mount prostate sample in a clinical specimen from a patient with cancer. Figure 2A shows a low-power, annotated image of the hematoxylin and eosin-stained histology slide, indicating the location of normal epithelium, epithelial hyperplasia, stroma, and cancer. In this experiment, the tissue section was incubated with an anti-PSA antibody and placed adjacent to seven analysis layers. Membranes 1, 3, 5, and 7 were coated with a PSA peptide, and membranes 2, 4, and 6 were coated with an irrelevant control peptide. The PSA antibody was then released from the tissue and passed through the analysis layers. As shown in Figure 2B, the antibody was specifically captured by the four PSA-coated membranes but not by the control layers. The measurements of the positive areas were done using an image analysis program. First, we compared and contrasted the stained areas among the four positive mem-

Figure 2. iLPA analysis of prostate tissue. A whole-mount prostate tissue (A) was incubated with anti-PSA antibody and captured by the membranes coated with PSA peptide (numbers 1, 3, 5, and 7) but not by the control layers (numbers 2, 4, and 6) (B). Intensity measurements of annotated areas appear in panel C.

branes and found that there was no lateral diffusion as the antibodies traversed the membrane stack. In other words, the four images could be essentially overlaid on top of each other. Next, selected histological areas containing different cell types were imaged, and the signals were quantitated (Figure 2C), with the epithelial components positive (hyperplasia-strong positive and carcinoma-weak positive for the PSA antibody) and the stromal compartment negative.

To compare iLPA measurements with standard IHC, the following experiment was performed. Three consecutive serial recuts of a whole-mount prostatectomy specimen were subjected to iLPA analysis, imaged, and were quantified. These same sections were then immunostained for PSA using standard IHC procedures (Figure 3, A and B). A comparison of IHC and iLPA results demonstrated that both techniques generate reproducible results, with experiment-to-experiment variance of 15 and 18%, respectively. The IHC method has an advantage of permitting detailed cellular and subcellular evaluation of staining patterns, whereas the iLPA method provides multiplex information on the regional "glandular-level" staining patterns. The dynamic range of the iLPA method appeared significantly greater than that of IHC, showing a 10.5-fold change between the weakly positive and strongly positive cells, as opposed to a 1.5-fold change in the IHC sections.

Figure 4 illustrates the application of the iLPA system to a TMA. Figure 4A represents the organization of the tissues. Two TMAs were used in the study; one was subjected to antigen retrieval before analysis, and the

Figure 3. Comparison of iLPA and IHC: prostate cancer. Three consecutive serial recuts of a whole-mount prostatectomy specimen were subjected to iLPA analysis, and these same sections were immunostained for PSA (A). A comparison of IHC and iLPA results showed that both techniques generate reproducible results (B).

other was not treated. In this experiment, cytokeratin 7 and PSA were applied to the sections, and iLPA was performed. Membrane 1 was coated with an irrelevant peptide, membrane 2 was coated with a PSA peptide, and membrane 3 was coated with a cytokeratin 7 peptide. The results are shown in Figure 4B. As expected, the control layer is negative, a positive signal for PSA is present only in the grid containing prostate cancer on membrane 2 (Figure 4C), and cytokeratin 7 is positive in the epithelial tissues (ovarian, breast, prostate, colon, and lung) in membrane 3 but not in brain, lymphomas, or melanoma (Figure 4C, grid). The signal is generally stronger after antigen retrieval as is demonstrated by the TMA on the right side of Figure 4B. To compare IHC and iLPA analysis of TMAs more thoroughly, an experiment similar to that shown for whole-mount prostate sections in Figure 3 was done. The results are shown in Figure 5, A and B. Both IHC and iLPA gave reproducible staining patterns, with more detailed histological evaluation possible by IHC and a larger dynamic range provided by iLPA analysis.

We next evaluated the measurement variance of the iLPA platform using a different tissue type. Table 2 summarizes a set of experiments performed on cryostat sections of minor salivary glands from nine Sjögren's syndrome patients and two minor glands from normal

Figure 4. iLPA analysis of a tissue microarray. Application of the iLPA system to a TMA showing specific capture of antibodies on their appropriate membranes. Panel **A** is a schematic representation of the different tissues on the TMA. Panel **B** represents the LPA membranes, and panel **C** is a magnification of the positive areas.

volunteers. Histological slides were incubated with a cocktail of antibodies including AQP5, M3, caspase 3, and cytokeratin 7, and the sections were washed and subsequently analyzed using eight peptide-coated layers. The membranes were scanned, and the signal intensity measured using ImagePro. Table 2 shows the average value and variance for each target antigen and each clinical sample. The data are drawn from 16 measurements per specimen. In each experiment, two serial recut tissue sections were included for each sample, there were two copies of each peptide-coated membrane in the analysis stack, and the experiments were repeated four times. The results indicate that the measurement variance of the iLPA system is relatively low and similar to that observed in standard immunohistochemical analyses as calculated in Figures 3B and 5B: 15% variance for whole-mount IHC and 25% for TMA IHC staining for the PSA antibody.

The multiplex capability of the iLPA platform was evaluated by examining expression levels of six different proteins in 12 different cellular phenotypes, including whole tissue sections and a TMA (Figure 6A). All tissues were incubated with a cocktail of glial fibrillary acidic protein, M3, AQP5, CD4, PSA, and cytokeratin 7 antibodies and subjected to iLPA analysis using 20 membranes. The layers included six peptide-coated membranes corresponding to the target proteins, and 14 albumin-coated

Figure 5. Comparison of iLPA and IHC: tissue microarray. A comparison of IHC and iLPA shows that both methods generate reproducible staining patterns (**A**), with more detailed histological evaluation possible by IHC and a larger dynamic range provided by iLPA analysis (**B**).

controls. The results are summarized in Figure 6B. In each instance, positive signals were observed in the expected tissue on the appropriate membrane(s). For example, the glial fibrillary acidic protein peptide-coated membrane was positive only for the brain tissue slide and the brain tumors on the TMA, and cytokeratin peptide-coated membrane 20 showed a positive signal in all of the epithelial tissues. A similar experiment was then performed using a Sjögren's sample and a TMA and analyzed by an iLPA capable of measuring seven analytes. In this instance, we used a prototype software system that allows the investigator to align, visualize, and measure the results. The visual output is shown in Figure 7A and the results summarized in Figure 7B.

In the long term, we envision that the iLPA platform will be easier to use and potentially more robust if "universal shuttle polymers" are used in place of individual antibody and peptide sets. A schematic of such a system is shown in Figure 8A. In this scenario, each membrane will have its own unique binding polymer that binds to it with high affinity based on a specific predetermined antibody-antigen pair. In addition, each polymer will have an open binding site so that it can be attached to a different primary antibody. In this way, the optimization of the polymer-membrane binding interaction will only need to be performed once, and investigators will subsequently be able to use the system for any primary antibodies of

Table 2. Summary of iLPA Results for Minor Glands

Minor gland no.	AQP5		M3 receptor		Caspase 3		Cytokeratin 7	
	Value	Variance	Value	Variance	Value	Variance	Value	Variance
1	0.187	0.038	0.089	0.059	0	0	0.166	0.027
2	0.169	0.043	0.057	0.069	0	0	0.155	0.057
3	0.11	0.078	0.04	0.04	0	0	0.178	0.048
4	0.173	0.033	0.049	0.06	0	0	0.15	0.021
5	0.0245	0.05	0.016	0.04	0	0	0.17	0.06
6	0.128	0.056	0.008	0.03	0	0	0.17	0.041
7	0.102	0.052	0	0	0	0	0.15	0.03
8	0.145	0.07	0.023	0.06	0	0	0.19	0.04
9	0.12	0.048	0	0	0	0	0.17	0.06
10	0.13	0.056	0	0	0	0	0.15	0.07
11	0.13	0.06	0	0	0	0	0.19	0.048

Minor salivary glands from nine patients and two normal volunteers were reacted with a cocktail of AQP5, M3, caspase 3, and cytokeratin 7. Contact transfer was performed on the tissues with a stack of AQP5, M3, caspase 3, and cytokeratin 7 peptide-coated membranes. The signal was quantified using the ImagePro program. The table represents a summary of four experiments performed with two tissue sections in every experiment and two membranes from every type in the stack.

interest by attaching them to the shuttle polymers. Moreover, the current requirement for a matching peptide antigen for each primary antibody in a study will be eliminated.

To evaluate feasibility of a universal system, the following experiment was performed. A prostate tissue section was placed on a glass slide and incubated with a combination of a murine-derived anticytokeratin primary antibody and a goat anti-human PSA antibody (as a positive

control). The section was then washed and incubated with a dextran polymer containing a goat anti-mouse secondary antibody and streptavidin. After incubation and washing, the primary antibody and the antibody-polymer conjugate were released from the tissue and subjected to iLPA analysis. The PSA antibody that served

Figure 6. Multiplex iLPA: multiple tissues. The multiplex capability of the iLPA platform was evaluated by examining expression levels of six different proteins (A) in 12 different cellular phenotypes (B).

B

intensity of signal	Minor salivary gland	TMA	ovarian	lymphoma	Breast	Prostate	colon	lung	brain	melanoma
Membrane 1-PSA	0	0	0	0	0.08	0	0	0	0	0
Membrane 2-GFAP	0	0	0	0	0	0	0	0.08	0	0
Membrane 3-M3	0.08	0	0	0	0	0	0	0	0	0
Membrane 4-AQP5	0.128	0	0	0	0	0	0	0	0	0
Membrane 5-CD4	0.082	0.06	0.062	0.058	0.061	0.065	0.063	0.067	0.065	0
Membrane 6-cytokeratin 7	0.168	0.068	0	0.068	0.066	0.077	0.07	0	0	0
Membrane 7-caspase 3	0	0	0	0	0	0	0	0	0	0

Figure 7. Multiplex iLPA: Sjögren's syndrome tissues and TMA. A Sjögren's syndrome sample and a TMA were analyzed by an iLPA capable of measuring seven analytes with the data analyzed in a prototype software system that allows alignment and visualization of the results (A). Quantitative signal intensity was measured by ImagePro (B).

Figure 8. Universal polymer iLPA system. The iLPA platform will be more versatile if universal shuttle polymers are used. A schematic of such a system is shown in **A**. Each polymer is bound with an antibody that is specific for one analysis membrane, and each polymer also contains an attachment site for a primary antibody (**A1**). The polymers are applied to a tissue section, and the primary antibody reacts with its antigen (**A2**). The polymer is then released from the tissue, binds to its specific membrane, and is measured (**A3**). **B** and **C**: Shuttle polymers can be successfully generated, bound to tissue, released, and analyzed by iLPA membranes.

as a positive control was detected on the PSA peptide-coated membrane 2 (Figure 8B, right) and showed a similar signal to the IHC staining for PSA (Figure 8B, left).

The antibody-polymer was successfully detached from the tissue section and captured by a biotinylated membrane as shown in Figure 8C (right), which is comparable with the cytokeratin 7 IHC image taken from a consecutive section (Figure 8C, left). This experiment demonstrates that shuttle polymers can be successfully generated, bound to tissue, released, and analyzed by iLPA membranes, a potentially useful advance for the next generation of the technology.

There are several different methods and technologies that can be used to accomplish the goal of multiplex analysis of tissue sections, including the new method described here. It is likely that many such methodologies will be useful in the molecular pathology field in the future, and each will have a unique utility depending on the needs of investigators. Multiplex protein analysis using IHC-stained sections is one approach for high-throughput measurements of expression for cells in tissues. There are several advantages to multiplex IHC analysis of serial sections; however, there are several disadvantages as well, including the time and effort required to prepare the multiple sections and the changing histology among serial recuts, especially the disappearance of important lesions such as preneoplastic tumor foci.

Alternatively, a multicolor immunofluorescence method using seven different antibodies was reported by Tsurui et al.¹² They used Fourier spectroscopy and singular value decomposition to produce seven-color analyses of immunofluorescence-stained tissue samples. To obtain the images, the authors used a combination of seven fluorescent dyes, three filter sets, an epifluorescence microscope, a spectral imaging system, a computer for data acquisition, and data analysis software. Quantum dot nanotechnology is another emerging and promising approach for multiplex analyses of tissue sections. The photostability of quantum dots allows repeated imaging of single molecules, and their size and electron density permits correlated electron microscopy.^{13–14} Ultimately, the strengths and weaknesses of each of these developing methods, including iLPAs, will need to be compared and contrasted, including cost, ease of use, and data quality.

In summary, presented here is a description of a prototype version of an application of layered peptide arrays. The technology combines two-dimensional histopathology with a third array dimension, thus permitting multiplex analysis of all cell types present in specimens. The hope is that iLPAs will help enable a global, systems biology approach to histological sections, integrating molecular data with morphology and thus generating new knowledge of disease states and processes.

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References

1. Visintin M, Meli GA, Cannistraci I, Cattaneo A: Intracellular antibodies for proteomics. *J Immunol Methods* 2004, 290:135–153
2. Braunschweig T, Chung JY, Hewitt SM: Perspectives in tissue microarrays. *Comb Chem High Throughput Screen* 2004, 7:575–585
3. Wang Y, Hayakawa J, Long F, Yu Q, Cho AH, Rondeau G, Welsh J, Mittal S, De Belle I, Adamson E, McClelland M, Mercola D: "Promoter array" studies identify cohorts of genes directly regulated by methylation, copy number change, or transcription factor binding in human cancer cells. *Ann NY Acad Sci* 2005, 1058:162–185
4. Furuya T, Ikemoto K, Kawauchi S, Oga A, Tsunoda S, Hirano T, Sasaki K: A novel technology allowing immunohistochemical staining of a tissue section with 50 different antibodies in a single experiment. *J Histochem Cytochem* 2004, 52:205–210
5. Chung JY, Braunschweig T, Baibakov G, Galperin M, Ramesh A, Skacel M, Gannot G, Knezevic V, Hewitt S: Transfer and multiplex immunoblotting of paraffin embedded tissue. *Proteomics* 2006, 6:767–774
6. Yu Y, Breitbart M, McNairnie P, Rohwer F: FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. *BMC Bioinformatics* 2006, 7:57
7. Sadlier DM, Ouyang X, McMahon B, Mu W, Ohashi R, Rodgers K, Murray D, Nakagawa T, Godson C, Doran P, Brady HR, Johnson RJ: Microarray and bioinformatic detection of novel and established genes expressed in experimental anti-Thy1 nephritis. *Kidney Int* 2005, 68:2542–2561
8. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA: Laser capture microdissection. *Science* 1996, 274:998–1001
9. Englert CR, Baibakov GV, Emmert-Buck MR: Layered expression scanning: rapid molecular profiling of tumor samples. *Cancer Res* 2000, 60:1526–1530
10. Gillespie JW, Best CJ, Bichsel VE, Cole KA, Greenhut SF, Hewitt SM, Ahran M, Gathright YB, Merino MJ, Strausberg RL, Epstein JI, Hamilton SR, Gannot G, Baibakova GV, Calvert VS, Flaig MJ, Chuaqui RF, Herring JC, Pfeifer J, Petricoin EF, Linehan WM, Duray PH, Bova GS, Emmert-Buck MR: Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am J Pathol* 2002, 160:449–457
11. Gannot G, Gillespie JW, Chuaqui RF, Tangrea MA, Linehan WM, Emmert-Buck MR: Histomathematical analysis of clinical specimens: challenges and progress. *J Histochem Cytochem* 2005, 53:177–185
12. Tsurui H, Nishimura H, Hattori S, Hirose S, Okumura K, Shirai T: Seven-color fluorescence imaging of tissue samples based on Fourier spectroscopy and singular value decomposition. *J Histochem Cytochem* 2000, 48:653–662
13. Giepmans BN, Adams SR, Ellisman MH, Tsien RY: The fluorescent toolbox for assessing protein location and function. *Science* 2006, 312:217–224
14. Jaiswal JK, Simon SM: Potentials and pitfalls of fluorescent quantum dots for biological imaging. *Trends Cell Biol* 2004, 14:497–504