

Layered-IHC (L-IHC): A novel and robust approach to multiplexed immunohistochemistry

“So many markers, so little tissue”

The need for multiplex detection of tissue biomarkers. There is an ever growing demand for increased biomarker analysis in human tissue specimens. Analysis of tissue biomarkers is key to understanding the activation status of particular pathways in specific cell types and can be indicative of disease diagnosis, prognosis, and prediction of therapeutic efficacy and monitoring of treatment outcomes. This information is of further importance in identifying and validating novel drug targets. While in certain instances detection of single biomarkers is sufficient, it is rapidly becoming clear that both panels of multiple markers and variations in biomarker levels relative to each other is more informative and more diagnostic of cell and disease status.

What is L-IHC? The ideal biomarker tests conserve precious tissue by multiplexing marker measurements, retain information regarding tissue morphology and cell type localization, and normalize marker levels to objective standards. 20/20's Layered-Immunohistochemistry (L-IHC) platform meets all of these requirements. The L-IHC technology platform also known as Multiplex Tissue Immunoblotting (MTI) was developed by 20/20 together with the NCI's Laboratory of Pathology under a Cooperative Research & Development Agreement (CRADA). In L-IHC the proteins of the tissue are released from a standard tissue section and captured on series of stacked membranes. The platform is based on specially constructed proprietary bioaffinity membrane layers. Tissue proteins are channeled upward through the membrane stack with each layer capturing a proportion of the entire protein complement while retaining the 2-dimensional spatial orientation of histologic areas of the tissue section. Thus each membrane represents a “carbon copy” of the tissue and can be probed for a different biomarker using standard immunoblotting techniques. Specific markers are detected using fluorescently labeled secondary antibodies and may be normalized to total protein. Advantages of L-IHC are listed in Box 1.

L-IHC can detect 5 – 10 Biomarkers per tissue section. L-IHC has been demonstrated to detect multiple biomarkers in a single tissue section (Figure 1). More than 25 biomarkers have been validated for use in L-IHC on a diverse set of tissue types (Table 1). Over 500 tissue specimens have been successfully analyzed by L-IHC. An important advantage of L-IHC over standard IHC is that because the antibodies are used to bind blotted tissue protein instead of binding directly to the tissue itself, many antibodies that do not function in IHC but work in western blotting can be used in L-IHC. This opens up the potential to monitor markers that have heretofore been inaccessible by standard IHC. It also often allows the same antibody that has been qualified in western blotting to be used for IHC.

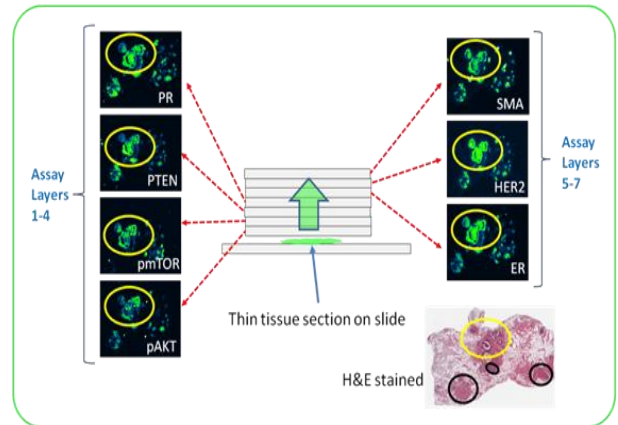


Figure 1: Normalization and reproducibility of L-IHC signal. (A) Relative intensity of total protein among 10 membranes. (B) Fluorescence images of Cy5-labeled total protein and FITC-labeled specific antigens. (C) Comparison of p-AKT signal determined from membranes 3 & 6 for a variety of cancer types. The bars represent the ratio of specific signal to total protein. Reproduced from Chung *et al.* Proteomics 2006;767-74.

Box 1: Advantages of L-IHC:

- Multiplexing of 5-10 markers per tissue section from routinely prepared FFPE and frozen tissue as well as TMA's
- Retains morphological information and cell/tissue type localization
- One layer can be reserved for an objective protein standard for normalization
- Quantitative fluorescence data allows for continuous-scale biomarker measurement
- Determines both protein expression level & extent of many protein modifications
- Advanced multifactor analysis enumerates & correlates complex relationships in cell signaling pathways
- L-IHC antibody panels may include antibodies often not useable in standard IHC allowing for measure of certain markers undetectable with standard IHC

Normalization of L-IHC specific signal. The transfer conditions for L-IHC have been optimized to afford consistent transfer of tissue protein throughout the stack of membranes. However, due to the inherent nature of the technique there is a gradual loss of protein from membrane to membrane through the stack, limiting the total number of membranes that can be used and requiring that there be a normalization of specific biomarker signals (Fig 2). Normalization is achieved by comparing specific signal to that of total protein on each membrane.

Agreement with standard IHC. L-IHC data compares favorably with that obtained by standard immunohistochemistry. Figure 3 shows a side-by-side comparison of standard IHC and L-IHC of HER-2 expression in a case of breast cancer. Note that L-IHC retains excellent resolution of corresponding histologic areas on the IHC down to the cellular level. In this experiment, proteins were eluted through 7 membrane layers and HER-2 was probed on membrane layer 4. The same resolution for probing biomarkers is retained in all layers. While L-IHC does not resolve intracellular structures (plasma membrane, cytoplasm, nucleus), this does not diminish its utility except in the rare instance when a change in the location of the biomarker is significant. The experiment displayed in Figure 4 investigated the expression of four biomarkers in a section of breast tissue encompassing a region of ductal carcinoma *in situ* (DCIS) as well as significant portions of normal breast tissue. One section was transferred to a stack of membranes and each layer was probed with antibodies against a specific antigen; including p-mTOR, p-AKT, estrogen receptor (ER) and HER2. Four other (non-consecutive) sections of this tissue were analyzed for each of the markers separately by IHC. The results obtained with all four markers by both L-IHC and standard IHC are presented in Figure 4 with a magnification of the primary area identified as DCIS by a pathologist. Note the strong correlation of the results from MTI for each marker with the areas that are stained in standard IHC.

Dynamic range of L-IHC. Quantitative image analysis is a vital element of tissue immunohistochemistry. Pathologists commonly grade immunohistochemistry in terms of both the intensity and distribution of staining for a given marker. Like standard IHC, L-IHC is capable of differentiating staining intensity levels, furthermore because quantification of L-IHC is a function of direct measurement of fluorescent intensity and utilizes a continuous scale making it far more objective than standard IHC. Staining for the HER2 biomarker in breast cancer is a primary example where staining intensity is of the utmost import. Typically staining is graded as 0 (weak), 1+, 2+ or 3+ where 2+ and 3+ are considered significant and warrant possible treatment with the drug trastuzumab. L-IHC has been demonstrated to successfully make this differentiation (Fig. 5). In this experiment a single slide contains breast cancer cores representing 1+, 2+ and 3+ staining as previously graded by a

Validated Biomarkers		Validated Tissue Types
Annexin 1	pan-Cytokeratin	Bile duct
c-jun	pan mTOR	Breast
Cytokeratin 7	Patched	Brain
Cytokeratin 14	phospho c-jun (S-63)	Colon
COX-2	phospho-AKT(S473)	Esophagus
EGFR	phospho-mTOR	Kidney
ER	PR	Liver
EZH2	pS6 Ribosomal Protein	Lung
HER2	PTEN	Lymph node
INT6	SMA	Ovary
Ki67	SPARC	Prostate
p300	TID6	
p53	vimentin	
Pan-actin		

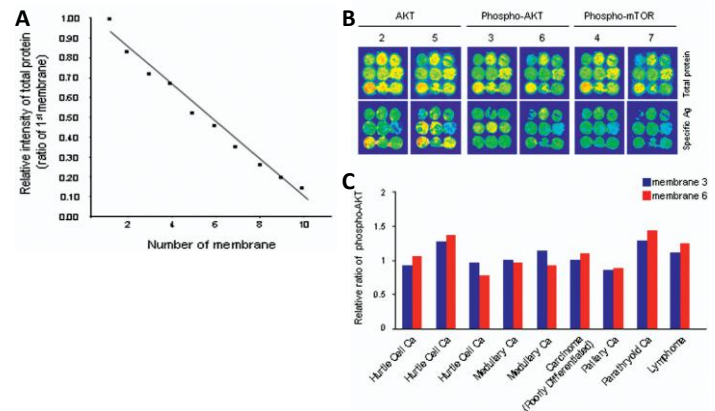


Figure 2: Normalization and reproducibility of L-IHC signal. **(A)** Relative intensity of total protein among 10 membranes. **(B)** Fluorescence images of Cy5-labeled total protein and FITC-labeled specific antigens. **(C)** Comparison of p-AKT signal determined from membranes 3 & 6 for a variety of cancer types. The bars represent the ratio of specific signal to total protein. Reproduced from Chung *et al.* Proteomics 2006;767-74.

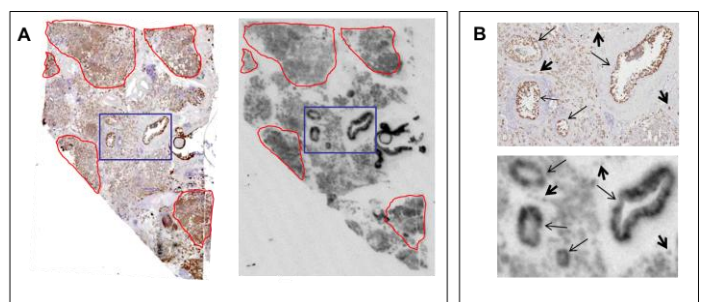


Figure 3: Breast tissue section containing cancer was probed for HER-2. **(A)** Left image shows IHC-stained tissue showing positive staining of ROIs (carcinoma) and the image on the right shows corresponding ROIs of fluorescent signal of the membrane (layer 4). **(B)** High-magnification of corresponding areas in the box (top, IHC; bottom, L-IHC) showing high resolution of DCIS (thin arrows) and individual cancer cells (thick arrows).

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pathologist. One slide was transferred to a stack of membranes and one membrane layer was probed with an anti-HER2 antibody. In parallel a separate slide was subjected to standard IHC. The superb correlation between L-IHC and standard IHC displayed in this experiment is indicative of both the comparability of L-IHC to IHC as well as the broad dynamic range for marker detection achievable via L-IHC.

L-IHC can be used to easily quantify and correlate multiple biomarker changes in a single tissue section to biologically relevant events.

In one study L-IHC was used to quantify the expression of seven biomarkers to investigate the normal to tumor transition of esophageal squamous cell carcinoma (Figure 6). Expression profiling of the different biomarkers was quantified using image analysis software. Briefly, five equivalent circular areas from each region were defined based on the tissue morphology as determined by H&E staining and the mean value of fluorescence was determined. Specific antibody signals were corrected for differential protein transfer and normalized to expression levels in either normal or stromal tissue. As can be seen in Table 2 changes in marker expression levels either up-regulation in the cases of COX-2, p53 and SPARC or down-regulation in the cases of CK-4, CK-14 and Annexin 1 correlate to the transition from normal to dysplastic to cancerous tissue. The expression level of the control Pan-CK biomarker remained constant over the three cell types.

L-IHC can probe post-translational modifications of proteins indicating pathway activation status.

L-IHC has been used to profile important cellular pathways. Figure 7 displays the mapping of the mTOR pathway in kidney cancer, specifically probing for p-mTOR, p-AKT, PTEN, p-S6 and pan-mTOR. While measurement of marker expression is highly relevant to following a cellular signaling pathway, expression level alone is often not indicative of a pathway's activation status. L-IHC is capable of monitoring the activation state of biomarkers by monitoring phosphorylation status. Because multiple markers can be measured in the same tissue section ratios of activated to total protein are more accessible through L-IHC than standard IHC. Furthermore, because marker determinations are performed on separated membranes rather than directly on the tissue, two (or more) antibodies against the same target (e.g. p-mTOR / total mTOR) can be used simultaneously avoiding issues of steric hindrance as might occur in standard immunofluorescence. Thus, Figure 7 further demonstrates the measurement of phosphor-mTOR and total mTOR and direct determination of the ratios of activated protein.

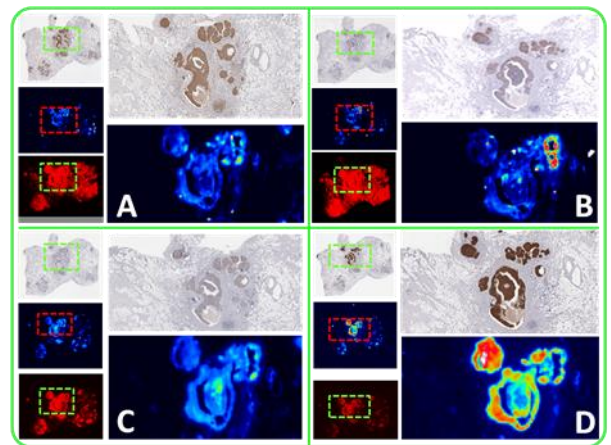


Figure 4: Comparison of L-IHC and standard immunohistochemistry for four markers in a breast cancer section. **A.** p-mTOR. **B.** p-AKT. **C.** ER. **D.** HER2. For each marker the three small panels depict immunohistochemical staining, specific marker signal in L-IHC and total protein signal in L-IHC, respectively. The larger panels are higher magnifications of the indicated areas.

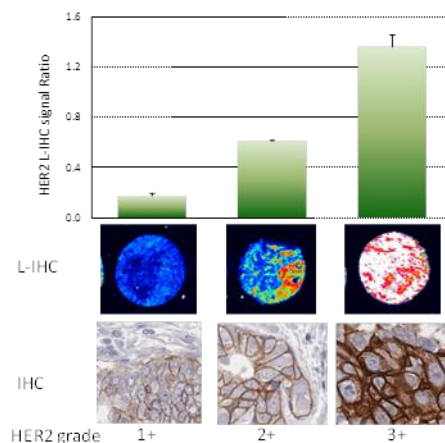


Figure 5: HER2 signal ratios as determined by L-IHC are well-correlated to HER2 grade level as determined by standard IHC. In comparison to standard IHC, L-IHC is more objective and yields continuous range of values.

Determination of disease prognosis by L-IHC. Recently, Chung *et al.* (Clin Cancer Res, 2009. 15(2): p. 660-7) performed a highly pertinent example of disease prognosis using quantitative multiplexed analysis of PTEN, p-AKT and p-mTOR ratios in extrahepatic cholangiocarcinoma (bile duct cancer) by L-IHC. Individual assay data were comparable to parallel IHC assays (Fig. 8). The technique revealed that decreased PTEN/p-AKT ($P = 0.003$) and PTEN/p-mTOR ($P = 0.009$) expression ratios were better predictors of patient survival than any single protein alone (Fig. 9). PTEN is an inhibitor of the pathway while p-mTOR and p-AKT are activated forms of the mTOR and AKT kinases directly involved in furthering the signal. In this case, Simply measuring the total expression level of a single biomarker is not necessarily indicative of a pathway's activation in a cell. If mTOR is highly expressed, but PTEN is also highly expressed the pathway may be inhibited. Thus, measurement of the ratios of two biomarkers such as PTEN to mTOR or of the activation state of the biomarker (e.g. p-mTOR / total mTOR; see above Figure 7) are likely to be more indicative of activation of the pathway. Thus, the utility of L-IHC for determining disease prognosis is clearly demonstrated. Standard IHC cannot provide quantitative ratio data. Thus, clinically useful data can be obtained using L-IHC, which enables simultaneous calculation of ratios among multiple targets that could not have been

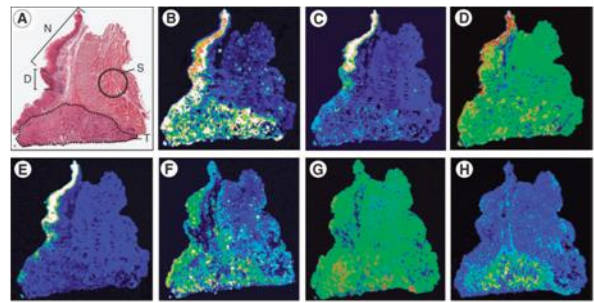


Figure 6: Biomarker expression profiling by multiplex tissue immunoblotting in human esophagus tissue. A. H&E-stained human esophagus. N, normal; S, stroma; D, dysplasia; T, tumor. Transferred membranes were incubated with antibodies against pan-CK (B), CK-4 (C), CK-14 (D), annexin 1 (E), COX-2 (F), p53 (G), and SPARC (H). Reproduced from Chung JY, *et al.* Cancer Epidemiol. Biomarkers Prev. 2006;1403-8

Table 2. Analysis expression levels of several biomarkers in esophageal normal, dysplastic and cancerous epithelium, and associated normal stroma

Biomarkers*	Type of tissue			
	Normal (n = 6)	Stroma (n = 6)	Dysplasia (n = 4)	Tumor (n = 6)
B Pan-CK	<i>1.00</i>	0.05	1.04	1.06
C CK-4	<i>1.00</i>	0.05	0.27	0.16
D CK-14	<i>1.00</i>	0.21	0.56	0.43
E Annexin 1	<i>1.00</i>	0.08	0.31	0.18
F COX-2	<i>1.00</i>	0.26	1.44	2.92
G p53	<i>1.00</i>	0.97	1.29	2.75
H SPARC	0.88	<i>1.00</i>	1.67	6.67

*The expression level of each antigen was normalized to expression either in the normal epithelium or in the stroma (bold italics), which was defined as 1.00.

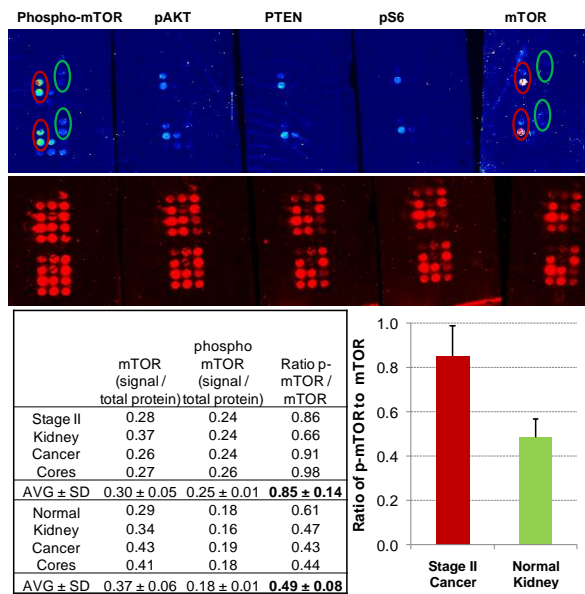


Figure 7: Measurement of marker ratios by L-IHC. **Top:** L-IHC data for 5 marker experiment using kidney TMA. **Bottom:** Calculation of relative marker levels by normalization of specific signal to total protein and comparing p-mTOR to total mTOR.

obtained with standard IHC imagery. This work is a key demonstration of the feasibility and practical application of L-IHC technology.

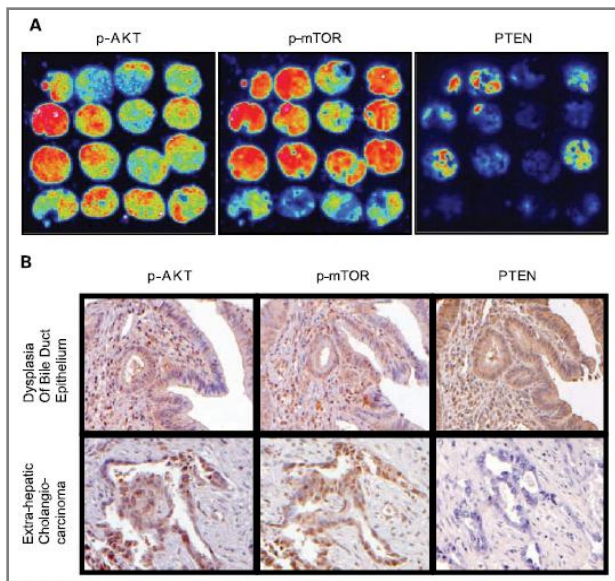


Figure 8: Phosphorylated AKT (p-AKT), phosphorylated mTOR (p-mTOR), and PTEN expression by multiplex tissue immunoblotting and immunohistochemical staining of a tissue microarray. A, lammyered membrane analysis; ratio of marker to total protein; ratio intensity from maximum to minimum is pseudo-colored as: white-yellow-red-green-blue-black in order. Cases with higher intensity to p-AKT and p-mTOR showed lower intensity to total PTEN. B, immunohistochemical stain of p-AKT, p-mTOR, and PTEN protein and dysplasia and extrahepatic cholangiocarcinoma. Reproduced from Chung *et al.* Clin Cancer Res, 2009. 15(2): p. 660-7.

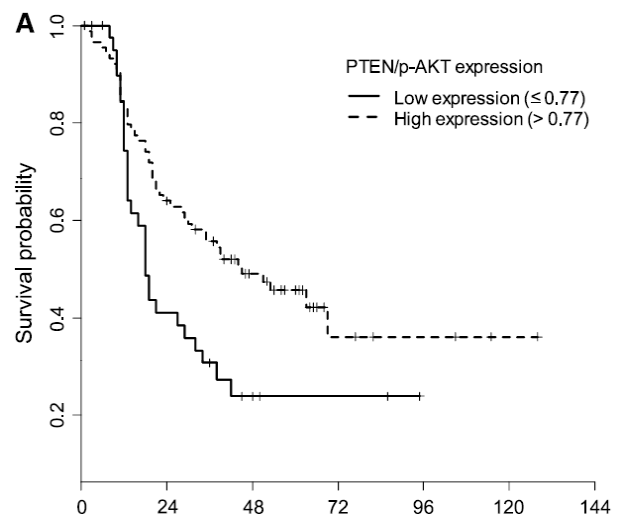


Figure 9: Kaplan-Meier survival analysis of extra-hepatic cholangiocarcinoma according to PTEN/p-AKT expression. Patients with low PTEN/p-AKT expression (median survival, 18 mo; n=42) had a significantly worse patients' survival time than those with high PTEN/p-AKT expression (median survival, 45 mo; n = 91; log-rank test, P = 0.003). Reproduced

Summary. L-IHC is a powerful technique for multiplexing immunohistochemistry. The method is robust, specific and reproducible and conserves precious clinical specimens. It is highly comparable to standard IHC while affording a broad dynamic range, continuous measurement scale and objective data analysis with internal normalization. Furthermore it allows for intra- and inter-experimental normalization, ratio-metric measurements and simultaneous determination of activated vs. total biomarker expression. The method is pertinent to pathway profiling, target validation, and clinical diagnosis; including prognosis, prediction of therapeutic efficacy and monitoring of treatment outcomes.