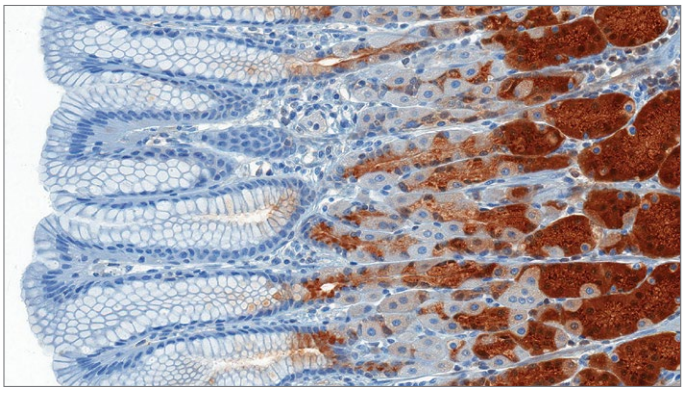

Immunohistochemistry for the 21st Century

Immunohistochemistry (IHC) dates back decades, but that doesn't mean it's stagnant. Automated sample processors, digital slide scanners, image analysis software, and more have given the aged procedure a decidedly 21st century flair. **By Jeffrey M. Perkel**

Immunohistochemistry is all about spatial relationships. If researchers want to know whether or not a given piece of tissue expresses a particular protein, they can use mass spectrometry or Western blotting. But to determine where in the tissue that protein is found, they have to visualize it in its cellular context.

That's where IHC comes in. Employing antibodies as probes for specific antigens, the method produces images of thin slivers of tissue—a tumor biopsy, for example—stained to display the expression and spatial distribution of one or more proteins.

"It answers the fundamental question of localization, of where you're going to find a particular target antigen," says Kevin Long, senior manager of the technical and scientific content development team at **MilliporeSigma** (a life science business of Merck KGaA in Darmstadt, Germany).

Spatial data can be used by researchers, for instance, to track lymphocyte infiltration at a site of disease; clinicians can use the data to grade tumors or assess a patient's likely response to a therapy.

The IHC laboratory at the **Naval Medical Center San Diego** (NMCSD) sees some 18,000 surgical cases a year, according to Director Greg Gates. Each yields anywhere from 2 to over 100 slides, depending on the size of the sample and the complexity of the case, but on average, Gates says, each case generates about 4 slides apiece—some 72,000 slides in all. Most pathology slides don't require IHC; about 80% of cases can be diagnosed from histologic staining (such as hematoxylin and eosin) alone, estimates Andreas Hoel, director of marketing for IHC at **Agilent Technologies.** But the remainder require more extensive evaluation.

Requiring from four to eight hours, the process is tedious but not complicated; starting with tissue on a microscope slide, the general process includes antigen retrieval, incubation with primary and secondary antibodies, washing, and development with a chromogenic reagent. The data are then interpreted using a light microscope.

"The best way I can describe IHC is, it's so easy a high school student can do it," says Stephen Hewitt, head of the Experimental Pathology Laboratory at the **National Institutes of Health** and editor-in-chief of the *Journal of Histochemistry and Cytochemistry*.

The problem is, each of these steps can be tweaked, and there is no one "standard" IHC protocol. As in many areas of biology, researchers don't always document their methods in sufficient detail for others to replicate them. And in any event, the resulting images are typically read by eye, an inherently subjective approach. As a result, variability from day to day and lab to lab can be considerable. But researchers have developed strategies for locking down that variability and improving other IHC pain points as well.

Automated sample preparation

One approach to decreasing IHC variability is using automation—employing systems that will treat every slide precisely the same, while at the same time freeing technicians and pathologists to tend to other tasks. "What we talk about as two pillars we improve on are laboratory efficiency and diagnostic accuracy," says Hoel, whose Dako Omnis system, for instance, automates both IHC and fluorescence in situ hybridization (FISH) staining.

The NMCSD pathology lab has purchased systems to automate two stages of the IHC process, says Gates: Sample processing systems from Polaris and Leica dehydrate and rehydrate the fixed specimens, while a BenchMark **Ventana** system from Roche automates the IHC staining itself.

For those with considerably lighter workloads—up to about 100 assays a week—the SNAP i.d. 2.0 IHC system from MilliporeSigma provides a vacuum manifold to increase the efficiency and reproducibility of manual slide staining, says Long. Researchers can process up to 24 slides simultaneously as reagents are removed from all slides at once via vacuum suction, he says.

Automation, says Hewitt, offers incontrovertible benefits—for clinical laboratories. But he notes that most academics engaged in basic research have little need for such power. After all, automation is just another word for robotics, he explains. "If you're just an investigator who needs to do a few experiments for your study, either find a good collaborator, or do it by hand."

Digital pathology

Another transformative development in IHC is the advent of so-called digital pathology. Instead of viewing and analyzing IHC slides under a microscope, digital pathology systems scan the stained slides in their entirety, storing the resulting whole-slide images in a digital format that can then be viewed and manipulated on a computer.

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That approach offers several benefits, says Catherine Conway, senior product manager for image analysis and pathology imaging at **Leica Biosystems,** which sells both brightfield- and fluorescence-based slide scanners under the Aperio brand. For one thing, whole-slide imaging facilitates medical education by ensuring that students have access to interesting and rare cases they might not otherwise see when sharing physical slides. But it also simplifies the process of sharing data with remote colleagues and obtaining second opinions—a process called "telepathology."

The **University of Pittsburgh Medical Center** (UPMC), for instance, offers a web portal through which distant researchers can upload slides for evaluation by the hospital's pathologists for between $50 and $150 per slide. Anthony Demetris, the Starzl Professor of Liver and Transplant Pathology at UPMC, who coauthored a 2012 study documenting the university's experience with telepathology, says he has evaluated samples from as far away as Australia and Asia. "It makes the interaction much more fluid than it would be otherwise."

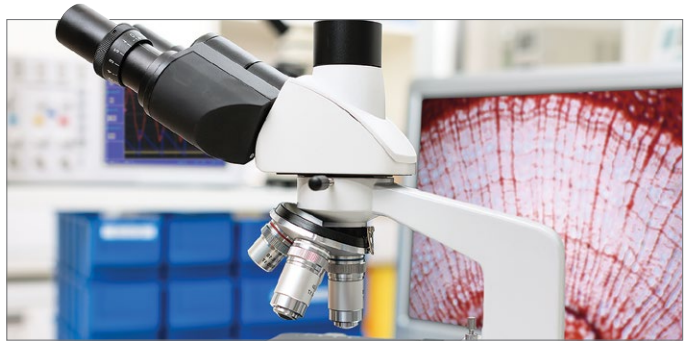
In one case, Demetris remotely evaluated a liver biopsy of an elderly patient that the primary physician suspected was simply inflammation. Demetris' review of the data suggested a tumor, so he asked the on-site physician to perform and upload some follow-up stains for T and B lymphocytes and Epstein-Barr virus (EBV) nucleic acids. In just a few days, he says, the proper diagnosis emerged: EBV-related lymphoma.

Whole-slide imaging also simplifies sample storage and retrieval by providing an easily accessible record of the slide (as opposed to physical slides), and it facilitates data analysis and subsequent reanalysis. "You can go back again and again to the same image and ask different questions," Demetris says. "It actually becomes a reusable data resource."

In addition to Leica, slide scanners are available from **Hamamatsu**, **Olympus, Philips,** Roche, and **Zeiss,** among others. But because each system uses a proprietary image format, researchers are generally restricted in what they can do with the images, especially if they have scanners from multiple vendors.

Mahadev Satyanarayanan ("Satya"), the Carnegie Group Professor of Computer Science at **Carnegie Mellon University** in Pittsburgh, Pennsylvania has had firsthand experience with that problem. Several years ago, Satya was working with pathologists to translate an image search algorithm he had developed to analyze digital slide images. He very quickly hit a wall—there was no way to search across different image formats. "The only software around that could interpret those formats was proprietary software," he says.

So, Satya's team worked out the details of the different file formats, codifying that knowledge in OpenSlide, an open-source C programming library. OpenSlide, he explains, functions like a computer printer driver, freeing programmers from needing to understand how a given scanner works. "You just say 'print,' and it's assumed that the device driver underneath will provide the appropriate translation to the specific idiosyncrasies of that printer." Similarly, programmers using OpenSlide can simply direct the software to read a file without having to know how it is actually structured.



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Researchers have used Open Slide to build other applications as well, such as telepathology portals and an educational resource called "SlideTutor." For his part, Satya has used it to implement his own search tool, called "Diamond," with which pathologists can ask such questions as, "Among all patients seen at this hospital between this date and this date who were taking this medication, do you see any [patients] where the following properties are similar to what I see in the slide?" OpenSlide, he says, "greatly increases the number of degrees of freedom you have as a designer."

Image analysis

Another benefit of digital pathology is the ability to perform sophisticated image analyses. Given a whole-slide image, these software packages identify cells and subcellular compartments (usually the cytoplasm, nucleus, and cell membrane), outputting such values as cell counts and staining intensity.

The Halo software package from **Indica Labs,** for instance, includes modules for counting cells expressing one or two proteins in the nucleus or cytoplasm, counting cells positive for membrane staining, assessing in situ hybridization, and more. Researchers can even assess spatial relationships, says Indica's chief scientific officer Kate Lillard. "For example, how many CD8-positive lymphocytes are within 50, 100, or 200 microns of the nearest tumor cells?" In one recent study, a research team led by Antoni Ribas at the University of California, Los Angeles used Halo to quantify lymphocyte subtypes within different tissue compartments in myeloma tumors. They observed a positive correlation between the presence of CD8+ cells specifically at the tumor margin with patient responses to antibodies targeting the "programmed death-1" receptor—data suggesting that it may be possible to increase the efficacy of such therapeutics by selecting the patients most likely to benefit from them.

Image analysis isn't new, of course, says Lillard—software for analyzing photomicrographs has been available for years. "The big difference here is that we're working on gigabyte-sized images." As a result, the software must be specially optimized to squeeze as much power as cont.>

Featured Participants

Agilent Technologies
www.agilent.com

Carnegie Mellon University
www.cmu.edu

Hamamatsu Photonics
www.hamamatsu.com/jp/en/5007.html

Indica Labs
www.indicalab.com

Laboratory of Pathology, NIH
ccr.cancer.gov/laboratory-of-pathology

Leica Biosystems
www.leicabiosystems.com

MilliporeSigma
www.emdmillipore.com

Naval Medical Center San Diego
www.med.navy.mil/sites/nmcsd

Olympus Life Science Solutions
www.olympus-lifescience.com/en

Philips Healthcare
www.philips.co.uk/healthcare/solutions/pathology

University of Pittsburgh Medical Center
www.upmc.com

Ventana Medical Systems
www.ventana.com

Yale Pathology Tissue Services
medicine.yale.edu/pathology/research/tissueservices

Zeiss
www.zeiss.com

Additional Resources

CellProfiler
cellprofiler.org

OpenSlide
openslide.org

SlideTutor
slidetutor.upmc.edu

possible from a computer's hardware. In an analysis of 50 whole-slide images, for instance, total Halo processing time ranged from 21.7 hours with one CPU core to 0.9 hours (64 cores).

A number of companies sell such tools, including Definiens (recently acquired by MedImmune), PerkinElmer, and Visiopharm. Alternatively, Anne Carpenter of the Broad Institute of the Massachusetts Institute of Technology (MIT) and Harvard has developed CellProfiler and CellProfiler Analyst, a pair of open-source software packages that accomplish many of the same tasks.

In theory, such tools could make life simpler in the clinic, for instance, by quickly flagging and counting cells and regions of interest. However, says Lillard, image analysis tools are used mainly in research and drug development rather than clinical laboratories, a result of regulatory obstacles. But that could change, she says. At the moment, researchers are using Halo and related software to identify interesting biomarkers that correlate with drug response or survival. But to be of use, they will have to translate those discoveries from bench to bedside. "The hope is that those will eventually transition into the clinic."

Quantitative immunofluorescence

Thanks to digital pathology and image analysis software, pathologists can score staining intensity not in relatively crude bins (0, 1+, 2+, 3+) as is done today, but as a continuous variable (say, 0–1000)—a change that should allow researchers to identify more subtle biomarkers.

Yet according to David Rimm, professor of pathology at Yale University and director of **Yale Pathology Tissue Services,** the traditional method of IHC isn't really compatible with continuous variables, and thus isn't truly quantitative. IHC, he says, "is not linear and it's not reproducible."

In traditional IHC, he explains, a secondary antibody conjugated to horseradish peroxidase converts a chromogenic substrate into a dark brown or red precipitate. But as light cannot easily penetrate that material—what Rimm calls "mud"—the method is at best semiquantitative.

Rimm's alternative is to use a fluorogenic substrate instead, a strategy called "quantitative immunofluorescence" (QIF). With such a strategy, small changes in protein abundance lead to comparable changes in light output, and coupled with whole-slide scanning and digital image analysis, that technique can provide a powerful platform for IHC analysis.

Breast cancer biopsies, for instance, are commonly tested for human epidermal growth factor receptor 2 (HER2) expression to determine whether the HER2-targeting drug Herceptin is likely to be effective. Herceptin binds the protein's extracellular domain, yet most of the antibodies used for HER2 IHC in the clinic target the protein's intracellular domain, notes Rimm.

In 2014 and 2015, Rimm and his team used QIF to assess whether they could detect a difference in staining with antibodies targeting the HER2 intra- or extracellular domains. As it turns out, they could. Using the chromogenic substrate 3,3'-diaminobenzidine and IHC, other teams found no observable difference between patients when using the cytoplasmic domain antibody and the extracellular domain antibody—staining intensity with both antibodies was identical. But using QIF, differences emerged. "About 10% to 20% of the patients' HER2 protein does not have any extracellular domain," he says. "So they're not likely to benefit from trastuzumab [Herceptin]."

Such seemingly minor fluctuations could make the difference between a successful drug and a failure, says Rimm. Referring to the staining categories used today, he says, "Science isn't ordinal"—it's continuous. And it's possible that the value of many potential biomarkers will only become apparent when using finer shades of intensity.

"Who knows how many other markers are out there that ultimately failed because there was not acceptance of the methods required to measure them quantitatively, and you needed to be above a certain level before the drug was effective?" asks Rimm.

That's not to say every histopathologist needs to switch to QIF and digital scanning. "Most of what pathologists do for IHC is, by design, nonquantitative," says Rimm. "It's 'fit-for-purpose.'" Indeed, IHC has been fit-for-purpose for over 70 years, with no apparent end in sight.

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