

Measuring Protein Expression in Tissue

The Complementary Roles of Brightfield and Fluorescence
in Whole Slide Scanning

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Reproducible protein expression measurement is important in many areas of tissue research and must address the inherently high biological variability across a single tissue section. Whole slide imaging is a new technology offering substantial improvements for both brightfield and fluorescence measurement of protein expression. In this article we discuss the latest developments in fluorescence and brightfield whole slide imaging as it applies to protein expression, and discuss how the 2 approaches differ and are complementary.

"I often think that the night is more alive and more richly colored than the day."
 -- Vincent Van Gogh

When a pathologist or biologist needs to measure protein expression in tissue, a choice must be made between immunohistochemistry (IHC), using brightfield microscopy with chromogens or immunofluorescence, using fluorescence microscopy with fluorochromes. With brightfield microscopy, the tissue is illuminated with visible white light, and protein expression is typically observed with 3,3'-diaminobenzidine (DAB), a brown stain. The darker the stain, the greater the protein expression. Counterstains and morphologic detail of the surrounding tissue help a pathologist study the tissue and draw conclusions. Is the protein expressed in the cytoplasm or the nucleus? Is the expression limited to cancer cells, or also present in the stroma? Alternatively, the tissue can be studied using fluorescence microscopy-observing 1 or more fluorochrome-tagged antibodies against a dark background, or against a counterstained background of a different color. Only the fluorochromes produce a signal, and over a working range, the signal is usually linear. A typical experiment would show a bright green signal

for 1 protein and a bright red signal for a second protein, and may show a relationship between the signals (**Image 1**). Pathologists and biologists have been using both brightfield and fluorescence microscopy to study protein expression in tissue for decades. Brightfield microscopy is generally the technique of choice, due to ease of use in a typical laboratory environment, as well as chromogen stability, and the ability to preserve the glass slides over time. Fluorescence microscopy, on the other hand, is usually performed in a darkened room. While the technique is both sensitive and specific, the specimen on the glass slide tends to fade after exposure to strong fluorescence illumination, and glass slides with aqueous mounting medium tend to dry up and degrade in quality over a shorter period of time. Going back to the fluorescent slide for additional analysis is often impossible. However, there is a new technology that puts the choice of methodology in a new light – whole slide scanning. Rather than capturing field-of-view images for small areas of tissue with a CCD camera, or “stitching” together individual contiguous images into a montage, whole slide scanning captures 1 mm swaths across the entire slide, using line-scan CCD camera technology. These swaths (or stripes) can then be aligned, stored, and accessed as an entire digital slide, from any location in the world, via the Internet. The impact of whole slide scanning on the laboratory workflow is substantial. A toxicologic pathologist in Europe can view and analyze a lesion in a rat kidney on a digital slide generated in China, or an urban clinical pathologist in Canada can overcome wide expanses of geography to diagnose a cancerous biopsy from a patient in a less well-served rural village. The concerns of long-term slide storage and fading of fluorescence disappear, as a rapid whole slide fluorescent scan gives a permanent, archive-ready digital image. Furthermore, a pathologist can use computer-based tools to identify cells or tissues, quantify protein expression, or perform image analysis across an entire tissue section in minutes. There is no need to limit the analysis to a few carefully chosen but potentially biased regions; protein expression can be analyzed on the entire tissue section (**Image 2** and **Image 3**).

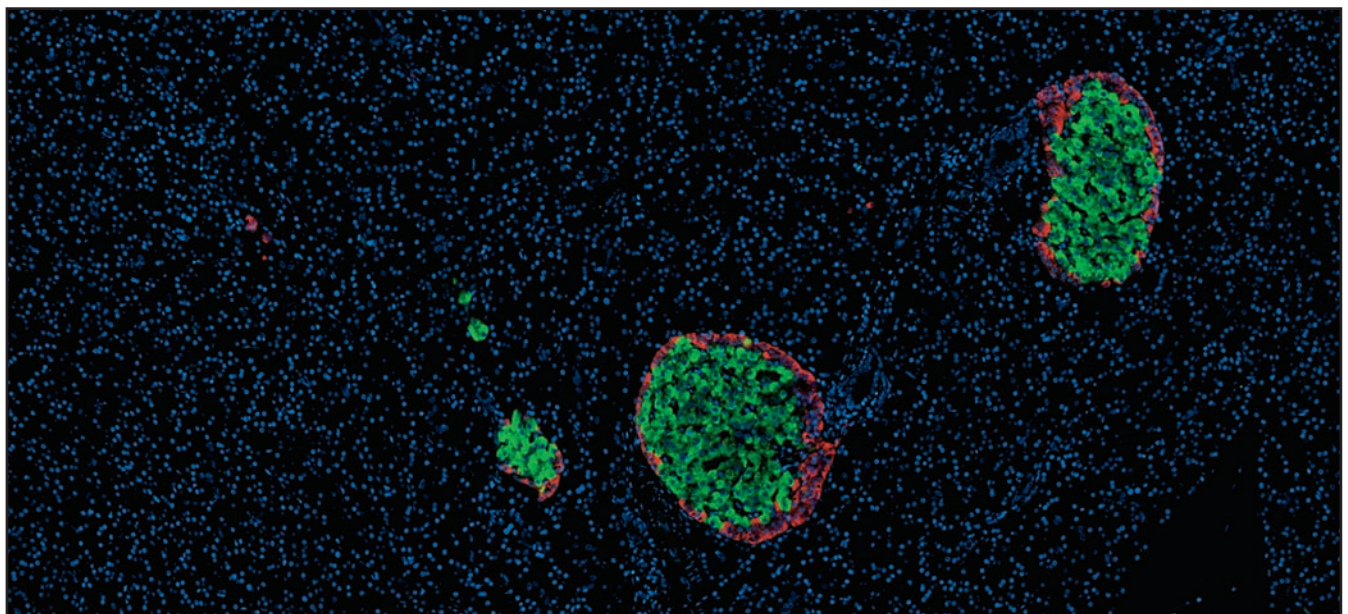


Image 1_Mouse pancreas section with islets stained in green and red, nuclei stained in blue.

Image 2_Rodent kidney section stained with hematoxylin and DAB (glomeruli).

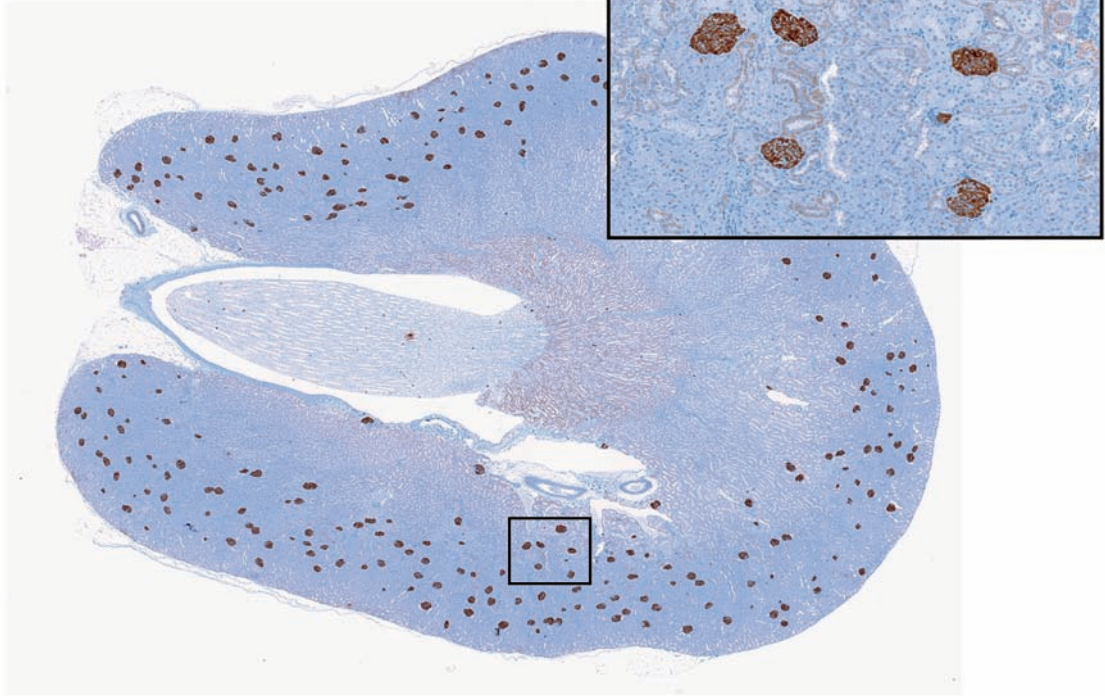
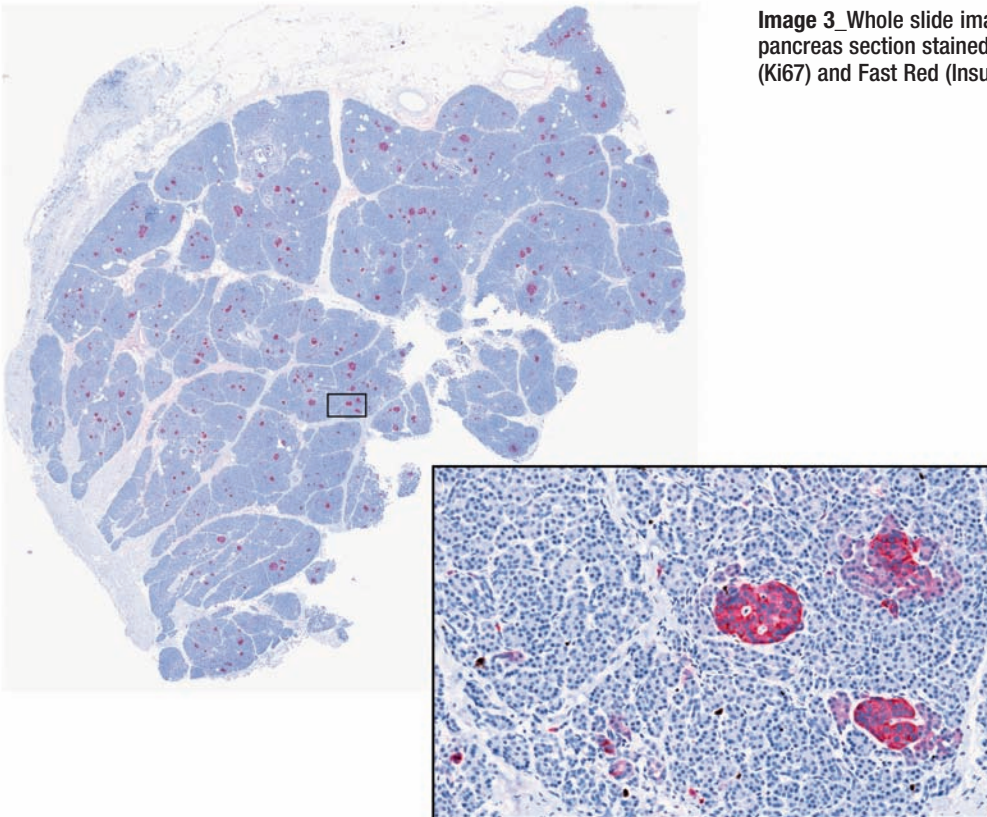


Image 3_Whole slide image and detail of human pancreas section stained with hematoxylin, DAB (Ki67) and Fast Red (Insulin).



With the availability of a much larger sampling area, it may be possible to measure protein expression more accurately than in the past. This continues the brightfield or fluorescence debate over which technique provides more quantitative protein expression in tissue. Much has been written recently denigrating the long-established art of immunohistochemistry in making protein expression measurements, in favor of immunofluorescence.^{1,2} However, when using whole slide scanning of tissue sections, brightfield and fluorescence microscopy complement each other.

Whole Slide Brightfield Scanning

With brightfield whole slide scanning, a stained histological section is placed on a motorized platform and scanned at 20X (or 40X) using a color line-scan camera. The stripes are precisely aligned, and the digital image is compressed to an acceptable file size for image sharing over the Internet or other network.^{3,4}

Protein expression is usually detected by indirect immunohistochemistry. Following unlabeled antibody binding to the protein of interest, a biotinylated secondary antibody (against the IgG of the animal species of the primary antibody) is applied, followed by streptavidin-horseradish peroxidase. This is reacted with 3,3'-DAB to produce a brown precipitate, indicative of the protein of interest. While a darker brown stain indicates more protein, the relationship of protein to signal is non-linear, due to non-linear signal amplification. Image analysis of the brown stain sometimes, but not always, requires color separation. Quantitation is usually done subjectively by a pathologist and scored as 0, 1+, 2+, or 3+. A primary example is the scoring of Her-2 protein, which is expressed (translocated/located on) the cell membrane. Cells with no membrane staining are classified as "0", while those with very dense staining are classified as "3+". Cells with a continuum of staining between these values are classified accordingly. These classifications can then be used to calculate a "score" such as the H-score⁵ or the Allred score, and these scores can be used to predict how a patient will respond to therapy. It is now possible to score Her-2 by computer-aided analysis.

Whole Slide Fluorescent Scanning

Fluorescent microscopy differs from brightfield microscopy in that it requires a specialized light source with a strong emission of wavelengths that excite particular fluorochromes. Individual filters or filter cubes are positioned in the light path to direct specific wavelengths to the specimen and to select for the longer wavelength fluorescent light emitted from the specimen.

Anyone who has done quantitative fluorescence microscopy has experienced the inherent challenges. While CCD cameras are linear devices, several factors can affect this linearity: exposure time must be limited to avoid saturation of the pixels; fluorescence photobleaching may occur during exposure and may affect results; lamp output may fluctuate and output wattage will decrease over time; tissue sometimes autofluoresces, or there may be non-specific fluorescence or cross-talk between the spectra of some fluorochromes. Despite these concerns, fluorescence is often the method of choice for co-staining the same cellular structures, or colocalizing 2 or more proteins. It is important that the design of a whole slide scanner for fluorescence applications take these factors into consideration to the extent possible.

Light Source

Potential fluorescence light sources include mercury, xenon, metal halide, and light emission diodes (LEDs). Each has advantages and disadvantages which revolve around output wavelength, output wattage, bulb/arc short-term and long-term stability, and bulb lifespan. An ideal whole slide fluorescence scanning light source would have: (a) high power output matching commonly used fluorochrome excitation wavelengths between 350 nm and 650 nm; (b) a long life cycle; (c) no need for alignment; (d) a computer-controlled light source; and (e) stable short-term and long-term output. The system must match the high frame rate performance of a high-end sensitive camera [ie, a time-delay integration (TDI) camera] with any fluctuations of the light source. Images acquired with capture rates of 6000 frames/sec and above usually exhibit these fluctuations. This eliminates the use of AC-operated light sources which might fluctuate up to 30% in amplitude. One good candidate for a light source for whole slide fluorescence scanning is the EXFO X-cite *exacte* (EXFO Life Science, Mississauga, Ontario, Canada), a highly stable, DC-operated, computer-controlled 200 W mercury light source requiring no alignment during installation. Moreover, it has a 2,000-hour life cycle that is almost 10 times longer than that of the commonly used 200 W mercury bulb for fluorescence microscopy. Light is transmitted to the system through a liquid light guide, thereby reducing the transfer of heat generated by the bulb to the sample. The bulb exhibits typical arc lamp light output degradation over time. However, for quantitative experiments, the degradation can be compensated with automatic adjustment of the light source iris. LED light sources also provide many advantages, but their output intensity is still not as high as the peak intensities of arc-based light sources such as the one previously mentioned.

Camera

For researchers interested in multiplexing using several biomarkers simultaneously, or in quantitation of fluorescence, sensitivity to a particular wavelength, bit-depth, and signal-to-noise ratio (SNR) are important camera characteristics. Every CCD sensor (chip) has a spectral profile indicating its responsivity to various wavelengths. The spectral emission of each fluorochrome must be taken into consideration when choosing a camera; for example, if 1 of the biomarkers is to be labeled with Cy5 (ex ~646 nm, em ~664 nm), the camera must be sensitive to wavelengths above 660 nm. A monochrome camera has greater sensitivity than a color camera. Most color CCD cameras have a Bayer mask on the chip, incorporate the use of red, green, and blue absorption filters in front of the chip, or use beam-splitters to split the light between 3 sensors, all of which reduce the amount of light hitting the detector. Therefore, when fluorescence signals are weak, there is a greater chance of them being detected by a monochrome camera. Furthermore, the ability to capture each color channel separately makes for easier quantitation, as a mask created from 1 channel (ie, DAPI-stained nuclei) can be applied to the images containing the antibody signal of interest, limiting the image analysis to the masked region, and allowing the researcher to determine how much of the antibody is localized to the nuclei. The bit-depth of the camera is also important, because a greater dynamic range provides more information for image analysis. Although most computer monitors are only capable of displaying 8-bit data (256 shades of gray), the raw data from a 10- or 12-bit (1,024 or 4,096 shades of gray) camera is preferred for image analysis purposes.

Motorized Hardware and Automation

High-throughput fluorescence operations require highly accurate and precise automation. With some scanner models, up to 300 slides can be queued, loaded, and unloaded onto the scanning stage. Various types of X-Y stages are available, controlled by stepper or servo motors, linear motors, or Piezo actuators, with linear motors more durable and quiet because they are frictionless. The microscope objective is mounted on a position-controlled Z-axis. Precise and coordinated control of the 3 axes is essential to provide a completely focused whole slide digital image. Closed loop position control with encoders as precise as 5 nm are often required for systems, and result in a scan resolution of 0.25 μm per pixel.

In order to maximize the amount of light coming through the system and achieve a high signal-to-noise ratio with good spectral separation of differentially-labeled biomarkers, it is important to use high-quality excitation, emission, and dichroic filters corresponding to the spectral characteristics of the fluorochromes. There are several ways in which the fluorescence filters can be physically positioned in the light path. Automated excitation and emission filter wheels and/or filter cube turrets can be synchronized to work together to change from 1 filter to another in as little as 0.5 sec. Scanning workflow typically starts with acquiring a macro image from the glass slide. Oblique or dark-field illumination can greatly increase the contrast in transparent fluorochrome-stained tissue sections, thereby facilitating tissue-finding.

It is extremely helpful if the fluorescent scanner can automatically calculate the optimum exposure time for each channel. Otherwise the operator must tediously employ a trial-and-error process of changing camera gains and exposure times for each channel, and scanning the sample several times. Aperio's (Vista, CA) fluorescence scanner, the ScanScope FL, provides an autoexposure routine that can be applied to all channels of the sample, with the option of manual entry.

The next step is to assign focus points (manually or automatically) on the tissue before initiating the scanning process. Auto-focusing is performed at these points in order to determine the sample topology and is repeated as the scanning progresses. For systems with monochrome cameras, only 1 channel can be scanned at a time. Combining a monochrome TDI line-scanning camera with an optimum filter configuration enables scanning of multiple channels for each stripe, and each channel of a single stripe is captured before moving to the next stripe. Using precisely aligned single-bandpass filter cubes, or a multi-bandpass filter set consisting of a multi-band dichroic mirror and a multi-band emission filter with single-band excitation filters for each fluorochrome, the resulting images can be optimally registered across all of the channels. As scanning continues, monochrome whole slide images are created automatically for each channel and can be fused together to provide a composite image (Image 4, Image 5, and Image 6).

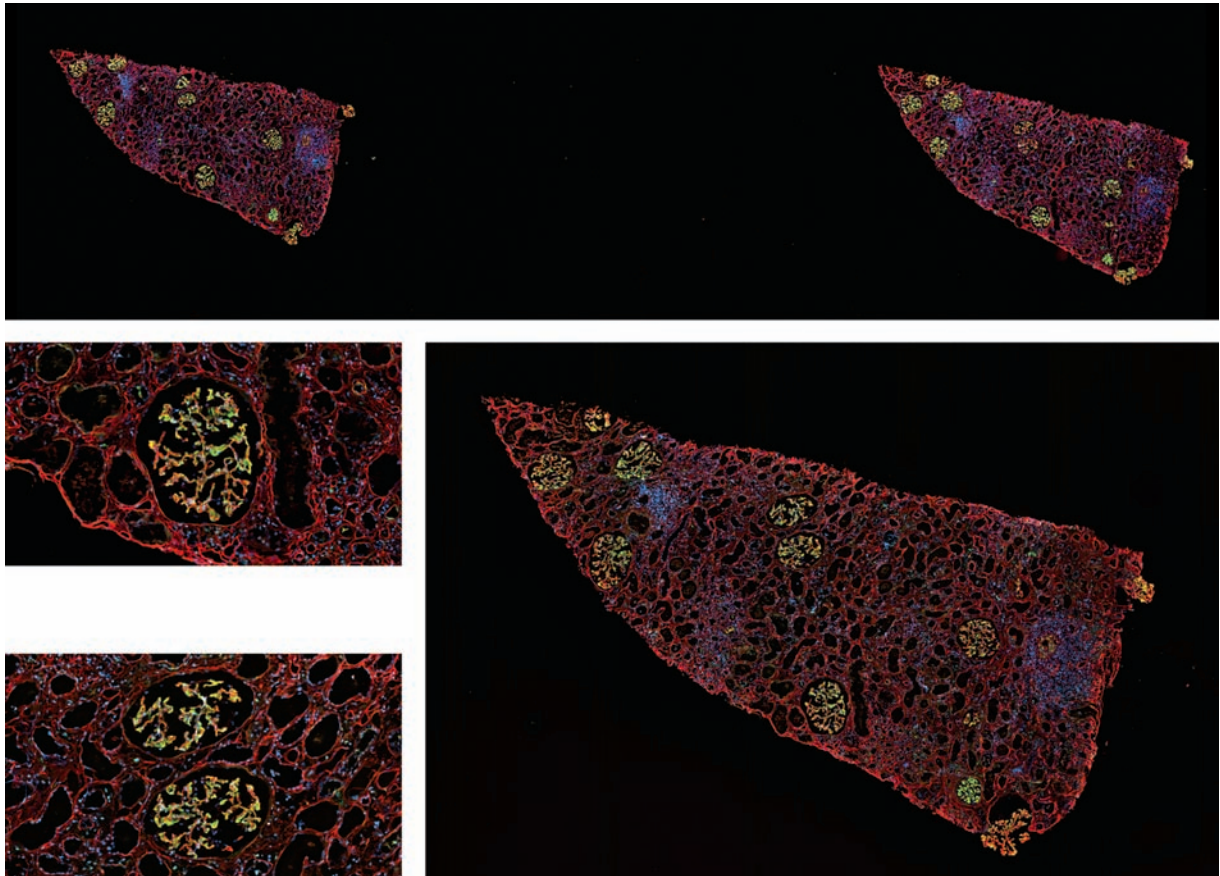


Image 4 Whole slide image and various views of 2 sections from a human renal biopsy stained with Cy3 (C4d, red), FITC (IgG, green), and DAPI (nuclei, blue).

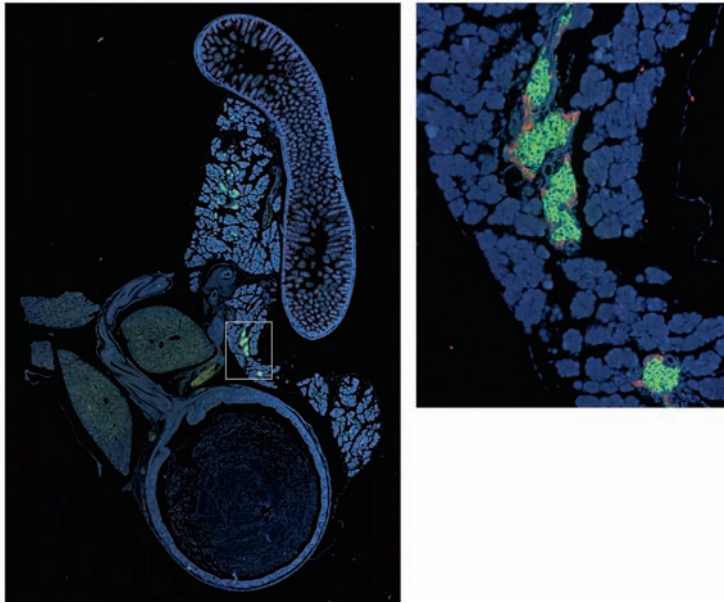
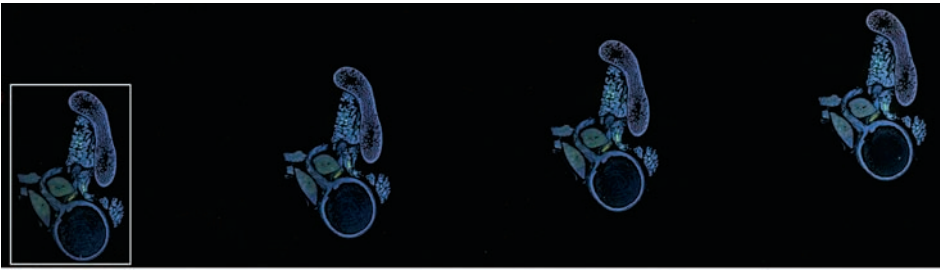


Image 5 Whole slide image and various views of mouse pancreas sections stained with Cy2 (insulin, green), Cy3 (glucagon, red), and DAPI (nuclei, blue).

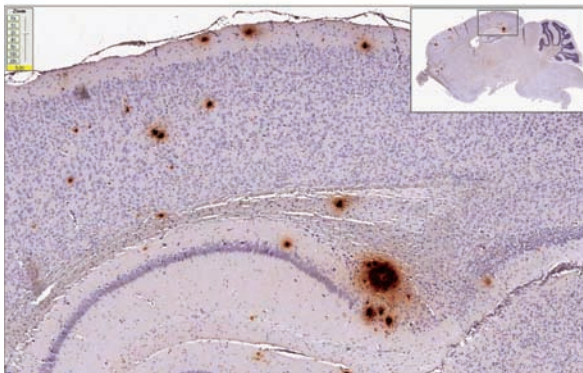
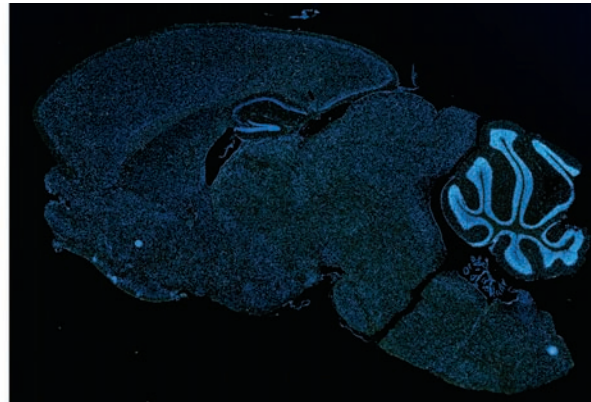
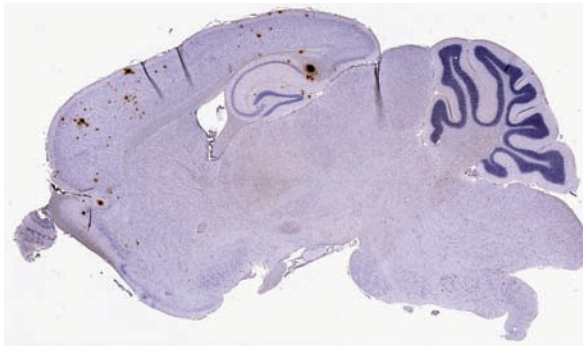


Image 6 Whole slide images of transgenic AD mouse brain sections stained for amyloid plaque: hematoxylin and DAB (6E10) on the left, and Hoechst 33258 (blue) and Thioflavin-S (green) on the right.

Where is the Protein Expressed?

It is often important to know *where* a protein is expressed in the cell not just *whether* it is expressed or how much of it is expressed. For example, the HER-2 *gene* is localized to the nucleus as seen with fluorescence *in situ* hybridization (FISH), while the HER-2 *protein* is localized to the cell membrane, as seen with IHC using DAB staining. In another example, β -catenin is a protein that displays complex subcellular localization and manifests oncogenic properties upon translocation to the nucleus. Nuclear-associated β -catenin activates several genes important in cell proliferation and invasion while membrane-associated β -catenin stabilizes cadherins-mediated cell-cell adhesion by facilitating the cytoskeletal attachment of adhesion complexes. Other biomarkers with important translocation properties include growth factor receptors EGFR, ERB-B4, and intracellular signaling molecules, and STATS. One novel approach to look at protein expression in tissue microarrays is called Automated Quantitative Analysis, or AQUA.⁶ First, a target protein of interest (eg, ER, β -catenin, growth factor receptors, and proliferation markers among others) is labeled with Cy5, and tissue-specific compartments are labeled with other specific fluorescent tags (Cy3-anticytokeratin for the cytoplasm, Cy3- α -catenin for the membrane, and DAPI for the nuclei). Using a fluorescence microscope with an automated stage, monochrome images of each fluorochrome channel are acquired, and tumor regions are identified using a mask derived from the ubiquitously expressed endothelial-specific antigen (cytokeratin or α -catenin). Images are analyzed using very specific image analysis algorithms to eventually produce an index of protein expression called an AQUA score.

Emerging brightfield approaches to protein expression use histology pattern recognition (eg, CRi Vectra, Definiens Tissue Studio, Aperio Genie) for the computer to recognize

microscopic tissue patterns after limited “training” by an operator. An individual tissue type can then be selected for subsequent image analysis with automated H scoring or similar approaches, without the pathologist making tedious region-of-interest drawings on the image.

High Intra-tissue Biological Variability Requires Whole Slide Imaging

Tissue presents an extremely complex architecture, and a cross-sectional tissue sample reflects this heterogeneity. Even in similar tissue types across a single section, the variability can be high. **Table 2** illustrates this variability across a number of tissue samples, where cellular signals have been measured at various locations in a single section, and then analyzed for their intra-tissue variability. A large study across 3 laboratories of 180 clinical breast cancer cases looked at composite variability of regions across individual tissue sections.⁷ Approximately 20

Table 2 High Intra-tissue Variability Requires Maximizing the Tissue Sample Area

Tissue	Count	Biological Variability Across a Tissue Section (Average Coefficient of Variation)
Human breast	HER2 (+3,+2,+1,0 scoring)	17%
Human breast	ER percent positive cells	11%
Human breast	PR percent positive cells	33%

Tissue cross sections exhibit high levels of biological variability. The table shows typical coefficients of variation by sampling multiple small related sections across a large tissue section, run by 3 pathologists in a controlled study using image analysis.

Table 1 Fluorescence vs Brightfield for Protein Expression Evaluation in Tissue

Aspect	Fluorescence vs Brightfield	Preferred Method
Observing protein expression in the correct tissue	Difficult or impossible to see tissue surroundings in fluorescence, without special stain for certain cells. Pathologists have years of training evaluating the correct tissue in brightfield slides.	Brightfield
Observing protein expression in the correct cellular organelle (eg, nuclear vs membrane vs cytoplasm)	Fluorescent stains generally better at specific cellular location (eg, stains like DAPI are stronger for identifying nuclei than hematoxylin in brightfield). Fluorescence also allows multiple organelle types to be stained (eg, 1 for the nucleus, 1 for the membrane). Brightfield is effective in membrane and nuclear protein expression, and can be used for cytoplasm staining in some situations.	Fluorescence
Minimizing distracting features	Only what has been stained positive is viewable in fluorescence. Other than autofluorescence, the background is largely eliminated.	Fluorescence
Training time for reading slides	Because the background is largely eliminated, it is easy to teach a technician to read fluorescent slides. Brightfield slides require years of histopathology expertise.	Fluorescence
Data analysis	With newer IHC quantitation tools, brightfield is not much more difficult than fluorescence now to analyze.	Even
Linear protein expression	Fluorescence is generally linear within a limited dynamic range. Chromogen staining is a non-linear reaction.	Brightfield
Reagent cost	Fluorochrome reagents more costly than chromogens.	Brightfield
Scanning cost and time	Fluorescent scanning can be more than 4 times longer than brightfield, and the equipment adds substantial cost.	Brightfield
Ability to detect low levels of expression	The non-linearity of chromogen staining allows for greater signal amplification.	Brightfield
Time sensitivity and archiving	Many fluorescent-dye signals fade over time, while the majority of chromogen dyes are very stable. Some phosphatase-based chromogen dyes can bleed over time. Problematic – fluorescent signal fades.	Brightfield
Multiplexing	In everyday practice, 3 or 4 are achievable without special technology (eg, quantum dots) in fluorescence; in brightfield, 2 antigens can be measured simultaneously (eg, a blue hematoxylin counterstain and red and black chromogens).	Fluorescence
Accepted for clinical use for protein expression	IHC brightfield assays are commonly used in breast cancer prognosis, with numerous FDA clearances by multiple vendors. Fluorescence has not been cleared yet for clinical protein expression, although it is widely used in FISH assays.	Brightfield

regions were chosen by a trained pathologist and then analyzed with image analysis to give an overall score for that tissue. The process was then repeated with 2 other pathologists, who worked with the same blinded sample and chose their own 15-20 regions and ran image analysis to give an overall score for each tissue. The variation ranged from a low of 11% for estrogen-receptor measurements to a high of 33% for progesterone-receptor measurements. This high intra-tissue variability was not due to the pathologist and will be inherently present regardless of whether the fluorescence or the bright-field approach is chosen. Since all of the regions analyzed represented similar tissue within the section (eg, only cancer cells were sampled), it clearly demonstrates the importance of whole slide imaging to facilitate sampling as much tissue on a slide as possible to generate a composite protein expression measurement per tissue section.

Conclusions

The high intra-tissue variability of protein expression requires the largest sample size possible per tissue, which is a key contribution of whole slide scanning. Whole slide image analysis techniques for quantitation in brightfield and fluorescent protein expression algorithms are continuously improving, as the hardware becomes more reliable. Both brightfield and fluorescent imaging have valuable contributions to make in protein expression studies. The greater use of whole slide imaging will lead to improvements in software quantitation, and in the future will significantly advance research tools used in the clinical laboratory. LM

1. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med.* 2002;8:1323-1327.
2. Gustavson MD, Bourke-Martin B, et al. Standardization of HER2 immunohistochemistry in breast cancer by automated quantitative analysis. *Arch Pathol Lab Med.* 2009;133:1413-1419.
3. Mulrane L, Rexhepaj E, Penney S, Callanan JJ, Gallagher WM. Automated image analysis in histopathology: A valuable tool in medical diagnostics. *Expert Rev Mol Diagn.* 2008;8:707-725.
4. Rojo MG, García GB, Mateos CP, García JG, Vicente MC. Critical comparison of 31 commercially available digital slide systems in pathology. *Int J Surg Pathol.* 2006;14:285-305.
5. McCarty KS, Jr., Miller LS, et al. Estrogen receptor analyses. *Arch Pathol Lab Med.* 1985;109:716-721.
6. Rubin MA, Zerkowski MP, Camp RL, Kuefer R, Hofer MD, Chinnaiyan AM, Rimm DL. 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.
7. Nassar A, et al. (2008). Trainable IHC HER2 Image Analysis System for Dako's HercepTest and Ventana's PATHWAY HER2. United States and Canadian Academy of Pathology (USCAP) Annual Meeting.