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The role and impact of quantitative discovery pathology

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The decision to advance an early-stage compound into formal preclinical testing depends on confidence in mechanism, efficacy and toxicity profiles. A substantial percentage of this confidence comes from histopathology interpretation, as the local tissue environment contains strong signals of both efficacy and toxicity. Accessing this tissue information is made difficult by biological variability across organs and tissues, an insufficient pool of pathology experts working in discovery, and the high subjectivity and individual isolation of microscope-based observations. This article describes how whole-slide imaging and quantitative analysis by trained pathologists are improving early-stage decision-making.

Introduction

Histopathological evaluations of animal tissues are an integral part of business decisions in drug development, especially early in the process, when key decisions are made at small biotech companies or pharmaceutical discovery groups for prioritization of good laboratory practice (GLP) preclinical testing. In science boardroom discussions, the advocate for the compound presents the pathologist's conclusions and the histopathological findings, often delivered by a consulting or internal pathologist who evaluates many studies with other candidate compounds. The advocate presents small representative images of pathologic changes or, if used, immunohistochemistry (IHC)-stained sections demonstrating the presence or absence of a biomarker protein to support the hypothesis. Unfortunately, the representative images are not typically any more characteristic of the pathologic change than a few hash marks would be of an entire football field.[1] The pathologist who performed the study is often not present and busy reading and preparing the results of the next study.

This common decision-making scenario in drug development illustrates three problems: a shortage of trained pathologists dedicated to discovery efforts, the biased individual isolation and selection of microscope-based observations, and the wide natural biological variability across single tissue cross-sections.

New digital pathology applications that provide whole-slide imaging and automated quantitative analysis are helping to address these problems and will become mainstream as the technology becomes more widespread at pharmaceutical discovery groups. With the use of digital pathology systems, the entire tissue section on a glass slide is scanned at high resolution, producing a large whole-slide image that can be stored in a secure Internet-accessible database. The two driving reasons for producing a whole-slide image are to eliminate geography barriers[2] and to make the entire tissue section available for quantitative image analysis.

Shortage of pathologists assigned to discovery efforts

Table 1 shows the distribution of employment of North American veterinary and medical anatomic pathologists in large pharmaceutical companies and contract research organizations. Within pharmaceutical companies, the industry average seems to be approximately one veterinary pathologist for every one billion dollars in annual sales, with one pathologist supporting on average ten research and development compounds. There is a strong consolidation of veterinary pathologists at several leading preclinical contract research organizations, where they are primarily supporting GLP studies. Several articles have described the impact that pathologists can have in discovery[3–6] if they have time available from their GLP preclinical work. Within some large pharmaceutical companies, there are discovery pathology groups (e.g. Discovery Toxicology, Investigative Pathology and Predictive Toxicology), enabling their preclinical pathologists to support early-stage discovery projects with 5–10% of their work time.

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TABLE 1

Employment of veterinary and human anatomic pathologists in the pharmaceutical industry.

Pharmaceutical company	Ethical drug sales[a] ($ millions)	Number of DVM pathologists[b]	Number of MD anatomic pathologists[c]	Number of R&D drugs[d]	Number of own drugs	Number of drugs under license
Pfizer	43,363	54	–	163	117	46
GlaxoSmithKline	36,506	36	–	249	157	92
Bristol-Myers Squibb	17,715	26	–	84	60	24
Merck & Co	26,191	19	–	173	115	58
Amgen	15,794	16	–	56	40	16
AstraZeneca	32,516	22	–	169	123	46
Eli Lilly	19,140	14	1	131	98	33
Wyeth	15,682	13	–	100	75	25
Novartis	36,172	17	3	165	99	66
Hoffmann-La Roche	30,336	21	4	191	123	68
Abbott	19,466	16	–	82	65	17
Sanofi-Aventis	35,642	20	–	160	103	57
Schering-Plough	14,253	8	–	86	40	46
Boehringer Ingelheim	11,595	11	–	54	38	16
Allergan	3502	6	–	34	21	13
Johnson & Johnson	29,425	9	1	141	83	58
Biogen-Idec	4097	5	–	41	20	21

Contract research organization	Sales ($ thousands)[e]	Number of DVM pathologists	Number of MD pathologists[f]
Charles River	1343	55	–
Covance	1827	50	3(?)
EPL		29	–
MPI Research		11	–
Huntingdon		10	–
WIL Research		8	–
Quest Clin Trials	177	–	35
Genzyme		–	

[a] 2008 Ethical drug sales, from 2008 company annual reports.
[b] A DVM pathologist is defined in this survey as an employee of a pharmaceutical company having either a veterinary medicine degree (DVM) and board certification (DACVP or ECVP). Compiled from the 2009 Society of Toxicology Pathology membership list, cross-referenced with the ACVP/ECVP membership lists. Only pathologists in North America and Europe were considered in this study.
[c] Data from an informal survey of pharmaceutical executives and managers, October 2009. Only anatomic pathologists in North America supporting pathology work clinical trials were considered in this study.
[d] Number of active R&D projects (July 2009) in early preclinical study through launch, compiled by Pharma Documentation Ring. Data from PharmaProjects (http://www.pharmaprojects.com/).
[e] 2008 sales from company annual reports. Data not listed for private companies.
[f] From company websites or annual reports.

The individual isolation of microscope-based observations

One of the primary roles of the pathologist is to effectively communicate histopathological study results to non-experts on the project team. The solitary nature of the microscope makes this difficult, especially when the discovery client teams are not closely co-located or the pathologist is part of a consulting group or a different organization. Solutions that have been implemented in pathology laboratories include multi-headed microscopes to enable fellow scientists and pathologists to view the same slide at the same time and having the pathologist take representative film or digital photos or images of areas of interest within a tissue section. Historically, these technologies have served well in providing information to others about what the study pathologist interpreted but were not without limitations. Viewing slides using multi-headed microscopes required all individuals to gather at the scope at the same time and spend considerable amounts of time to view the images. Conversely, looking at representative digital or film images allowed individuals to view them at their selected times and in varying location; although this allowed some flexibility in viewing the images, they were forced to rely on the pathologist to take accurate representative images and were further hindered by not discussing the results in a real-time environment.

Wide natural biological variability across tissue sections

Although the local tissue environment can provide remarkable insight into both efficacy and toxicity of chemical agents, tissue presents an extremely complex architecture with variable heterogeneity within most tissues. Even in similar tissue types across a

single section, the variability can be high. Fig. 1 illustrates this variability across several tissue samples in different internal studies by the authors, in which cellular signals have been measured at various locations in a single section and then analyzed for their intra-tissue variability. For example, coefficient of variation in a given xenograft for randomly sampled regions of manual microvessel counting averaged 46% and automated area measurements averaged 37%. Neutrophil and lymphocyte counts in rodent livers had lower intra-tissue variability but still higher variability than one would expect in more homogenous samples such as urine or serum. In individual human breast tissue sections, average variability ranged from 10% to 63%. The variability of single regions in a section is clearly high across a tissue section. A much larger study used for FDA clinical submission looked at composite variability (Fig. 1) of regions across individual tissue sections.[7] In this case, 15–20 regions were chosen by a trained pathologist and analyzed with image analysis to give an overall score for that tissue. The process was then repeated with two other pathologists, who worked with the same blinded sample, chose their own 15–20 regions and ran image analysis to give an overall score for each tissue. The variation with a composite score is generally much reduced but still ranged from a low of 11% for estrogen receptor measurements to a high of 33% for progesterone receptor measurements.

This variability is not a reflection of the pathologist's capabilities but illustrates the challenging and heterogeneous nature of tissue samples. Because all of the regions analyzed represented similar tissue within the section (e.g. only cancer cells were sampled), it leads to the logical conclusion that it is important to sample as large a region as possible in a tissue section, provided the regions all contain the appropriate target tissue. This variability is leading the industry into the increased use of stereological principals to ensure representative sampling.

Whole-slide imaging

Paralleling the shift from processed sheet film to digital images in radiology departments, pathology departments are also – albeit slowly – making the change from glass slides to whole-slide digital images viewed on a computer monitor. The technology for scanning an entire tissue section on a glass slide has been reviewed previously for its utility in drug development.[8,9] For the first time, investigators and other pathologists are provided full transparency of a complete tissue section, either viewed at their leisure or with the capability of communicating real-time with the pathologist, via web-based conferencing or internal customized image sharing. Providing complete tissue images for viewing and analysis saves time and empowers investigators to take a more active role in identifying or communicating the areas and possible lesions of interest to the pathologist, saving countless hours of pathology time trying to find what the investigator is looking for.

An extremely valuable practice is providing digital images to researchers and, after reviewing the basic anatomical features of target tissues, letting investigators sort through the digital images and come back to the pathologist with questions and/or potential conclusions. This not only frees up the pathologist's time from sitting with a researcher and reviewing tissues using a multi-headed scope but also encourages the researchers to study closely the images in support of their hypothesis. An added benefit is that the researcher spends the time to examine specifics within the tissue without having to repeatedly return to the pathologist for clarification. Although final conclusions are always the responsibility of the pathologist, the interactions with digital slides greatly increase researcher–pathologist interaction, engage researchers and help them to understand the nuances of tissue variability.

Image analysis in histopathology

When given a choice, investigators almost always prefer quantitative efficacy data from their pathologists to the usual qualitative pathologist grades (e.g. minimal, mild, moderate and marked). Hence, image analysis of digitized images provides a practical quantifiable means of measuring cellular change and, consequently, replaces subjective with objective evaluation. One can divide histopathology image analysis into three basic overlapping approaches: area-based measurements, cell-based measurements and object-based measurements (Fig. 2). Area-based measurements, in which stains are isolated from each other, can be useful in measuring areas and intensities of various color hues in target tissues. Every stain, such as hematoxylin, eosin or diaminobenzene (DAB), comprises different proportions of the primary color (red, blue or green) elements. The user defines the stain of interest, either as hue on a color wheel ranging from 0 to 1 (e.g. 0.1 = DAB, 0.33 = green, 0.66 = blue, 1.0 = red), or by entering RGB decimal values. Area-based approaches are useful for measuring a single, visually apparent substance in tissue regardless of color and have the advantage that hue width and threshold can be precisely adjusted to 'digitally see' what the pathologist sees on the slide. The color is binned into three or more grades or levels of intensity (e.g. 0, 1+, 2+ or 3+ or low, medium, high) and a count is made of the area occupied by each in terms of either pixels or μm^2.

Color deconvolution is a widely used image analysis process that digitally separates each histology stain.[10] The great advantage is that a stain of interest is accurately measured and is not partially obscured by other stains on the slide. In other words, the algorithm can see what the pathologist does not see on the slide. An example of its use would be a case in which DAB is not obscured by hematoxylin in an IHC stain. Another example would be a case in which red coloration of a periodic acid Schiff reaction for glycogen would not be obscured by hematoxylin in a liver section.

Cell-based analysis measures RGB values inherent in stains such as DAB or hematoxylin and typically focus on either nuclear or membrane stain affinity. In nuclear stains, each nucleus is identified by the intensity of a counterstain (e.g. hematoxylin) and each cell is assumed to contain one nucleus. Negative and positive nuclei can be determined and counted. Nuclei can also be simultaneously categorized and segregated by size, shape and/or stain intensity, as reflected in cell type, cell-cycle stage or cancerous nuclear effects. This capability can be useful in characterizing changes such as cellular hyperplasia or hypertrophy, cellular infiltration, nuclear pleomorphism and tabulation of specific cell types within a tissue. An example of its use might be to determine percent and degree of DAB positivity of neoplastic cell nuclei. Stromal cell nuclei, which results in false negative nuclear counts,

Tissue	Count	Number of sections sampled in a tissue	Number of tissues	Biological variability across a tissue section (average coefficient of variation)
Variation of individual regions across a single tissue section				
Mouse xenografts	Microvessel count (manual)	10	9	46%
Mouse xenografts	Microvessel area (automated)	10	9	37%
Mouse livers	Neutrophil counts	8	2	16%
Rat livers	T lymphocyte counts	21	2	32%
Human breast	HER2 H Score	7 to 11	5	10%
Human breast	ER H Score	6 to 10	5	41%
Human breast	PR H Score	6 to 11	5	63%
Variation of different composite regions averaged across a single tissue section				
Human breast	HER2 (+3,+2,+1,0 scoring)	15-20 averaged as one composite region	180	17%
Human breast	ER percent positive cells	15-20 averaged as one composite region	180	11%
Human breast	PR percent positive cells	15-20 averaged as one composite region	180	33%

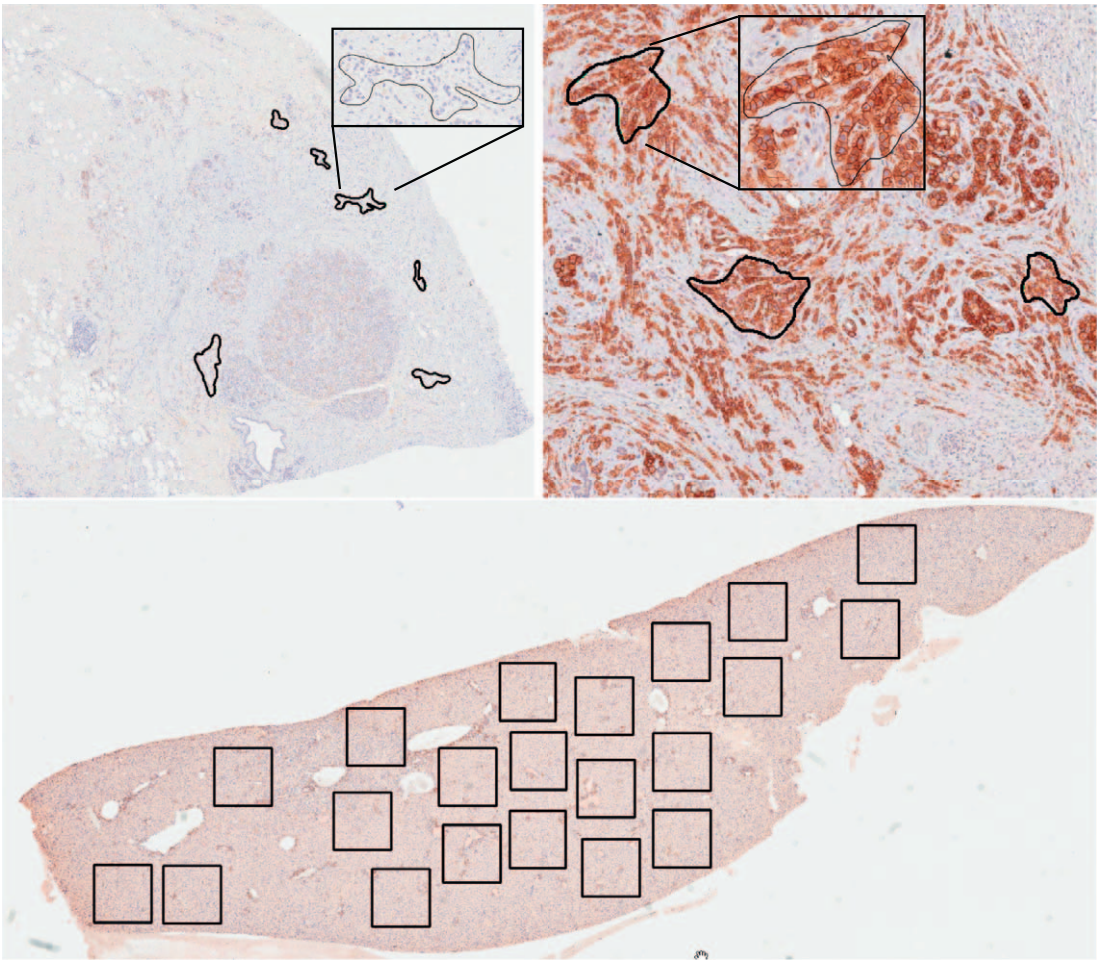

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FIG. 1

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. Typical sampled regions are shown for a human ER breast cancer tissue at middle left, a HER2 breast cancer tissue at middle right, and a rat liver sampled at bottom for T lymphocytes.

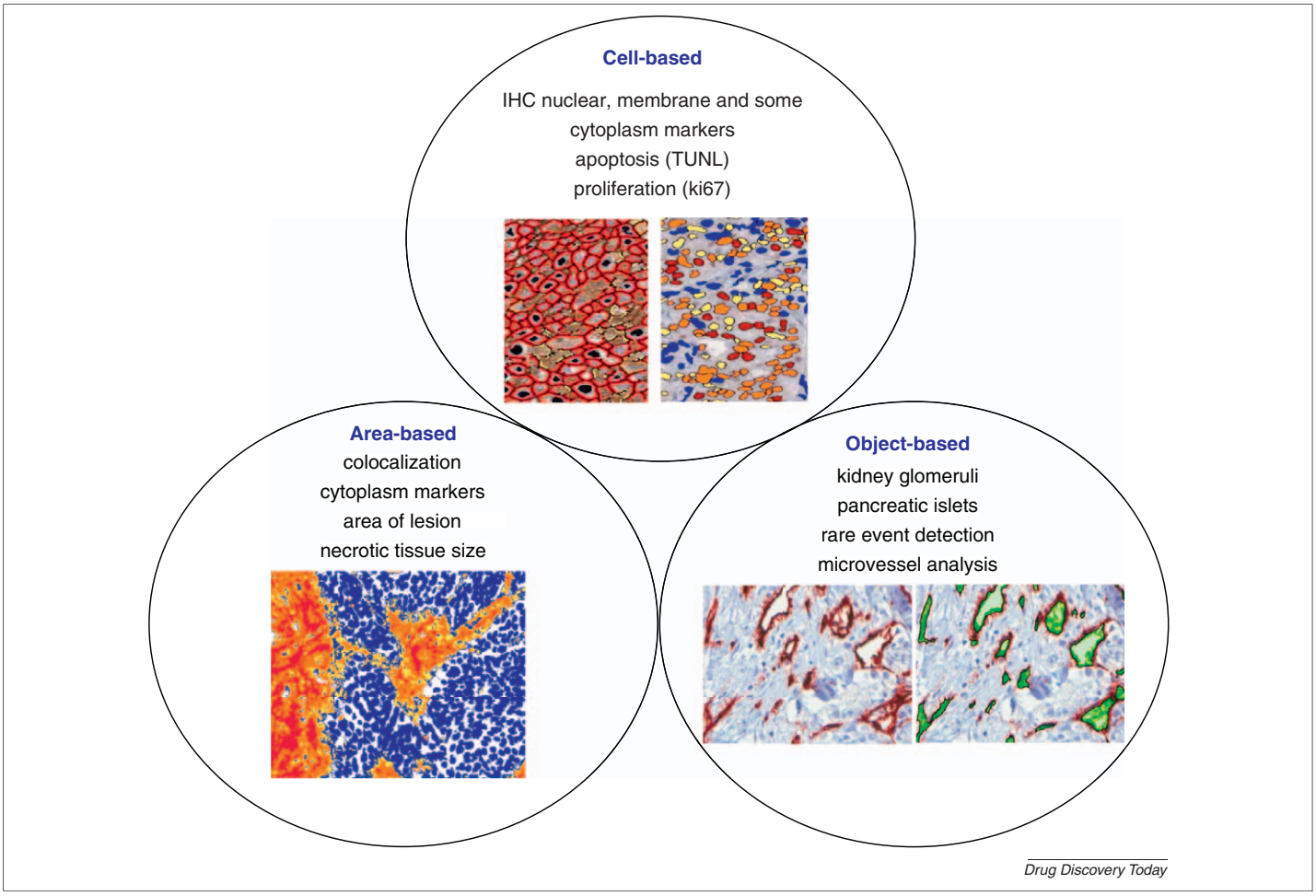

FIG. 2

Image analysis measurements can be classed as cell-based, area-based, or object-based.

can be excluded if shape and size are substantially different from adjacent neoplastic cell nuclei. More advanced image analysis tools, such as proximity measurements, are required to exclude stromal nuclei with shape and size more similar to neoplastic cell nuclei. Other examples might include measuring hepatocellular hypertrophy (nuclear number/unit area of hepatic parenchyma) and measuring numbers of infiltrating inflammatory cells per unit area in a given tissue.

With membrane analysis, the nuclei are also identified with a nuclear counterstain, to assist in finding cells, and individual cell membrane staining is then quantified for intensity and completeness of the membrane stain. Although most commercially available software programs for membrane analysis have been optimized for the clinically widely used human epidermal growth factor receptor 2 (HER2)-stained breast tissue measurements, they can also be used for other tissue types and membrane stains. Cells are classified as 0, 1+, 2+ and 3+ based on their membrane stain intensity, with completeness and percentages of each integrated into a standard HER2 scoring scheme. The longstanding solution to estimating marker expression in both nuclear and membrane analyses is a subjective scoring system using a convention called the 'H-score' method.[11] With this method, the evaluation scores staining features of cells (e.g. cytoplasmic, nuclear or membranous staining) by intensity of stain according to grades of 0, 1+, 2+ or 3+ and assigns relative percentages of the tumor cells having each grade. Accordingly:

$$H\,\text{score} = (1) \times (\%1+) \times (\%\text{Area}) + (2) \times (\%2+) \times (\%\text{Area}) + (3) \times (\%3+) \times (\%\text{Area})$$

$$\text{Example}: (1) \times (+5\%1+) + (2) \times (65\%2+) + (3) \times (15\%3+) = 180$$

With image analysis, the thresholds that define the intensity levels are programmed into the analysis algorithm (usually with pathologist review and input) and an H-score calculation can be consistently applied across various samples.

Object-based measurements are typically more complex than area- or cell-based measurements. In this approach, individual cells are grouped into a single object for measurement and computation of object statistics. For example, a cross-section of a microvessel might contain multiple endothelial cells arranged around a lumen. In this case, what is of interest is the microvessel object, with the identification of positively stained endothelial cells only an intermediate step to finding the complete object. In microvessel analysis, a stain for endothelial cells (e.g. CD31 or Factor VIII) is coupled with color deconvolution methods to identify endothelial cells, and then the algorithm is programmed to identify vessel-like objects.[12] The user can automatically filter

out existing larger vessels and identify and compute morphology statistics on only smaller angiogenic vessels. Output statistics include individual and mean vessel area, vessel perimeter, lumen area, vascular area and vessel wall thickness computed on each vessel. A common application of microvessel analysis is in classifying vessel types and quantitating degree of angiogenesis in a mouse xenograph neoplasm. There are many other potential object-based measurements, including micrometastasis analysis for locating rare tissue events, such as tumor metastases, or identifying amyloid plaque objects in Alzheimer rodent models.

Whole-section analysis with histology pattern recognition

Whether the approach is to measure area, cells or objects, one of the biggest challenges of obtaining accurate image analysis results is the segregation of target tissue (region-of-interest) from other tissues on a slide. Before whole-slide imaging, one commonly captured a dozen representative images of small square areas that contained only the target tissue with a microscope-mounted camera (film and then digital), then performed evaluations and/or image analysis on these areas. The use of whole-slide imaging enables one or two orders of magnitude more tissue to be analyzed because the image analysis can be conducted across the entire tissue.

However, whereas choosing a few representative regions puts the burden on the pathologist to choose appropriate locations, with whole-slide image analysis the burden is again on the pathologist to analyze the appropriate tissue on the section. To do this manually it requires tedious manual separation of regions of interest, which can be ineffectively time consuming. To solve the challenge of target tissue specificity, various software vendors (Visiopharm, CRI Vectra™, Definiens Tissue Studio™ and Aperio Genie™) have introduced automated pattern recognition software as a preprocessing utility to segregate target and nontarget tissue during analysis (Fig. 3). The basic concept is for the computer to recognize microscopic tissue patterns after training by an operator. An individual tissue type can then be selected for subsequent image analysis, without the pathologist making tedious region-of-interest drawings on the image. It thus serves as a preprocessing utility for subsequent analysis by any of the cell-, area- or object-based approaches or can be used independently to calculate various areas of the different tissue types across a slide.

In cancer applications, pattern recognition enables measurements such as total neoplastic cell area, total numbers of neoplastic cell nuclei, mean nuclear size and mean neoplastic cell size. There is also great potential for the use of pattern recognition software in toxicologic pathology. Examples in hematoxylin & eosin (H&E)-stained sections include extramedullary hematopoiesis or periarteriola lymphoid sheaths of mouse spleen, hepatocellular necrosis or bile duct hyperplasia of rat liver, follicular cell hypertrophy of rat thyroid gland and myocardial fibrosis or inflammatory cell foci of mouse heart. In diabetes research, the ability to successfully analyze tissue components of the pancreas is a valuable asset to investigators.[13,14] Pancreatic islets and acinar

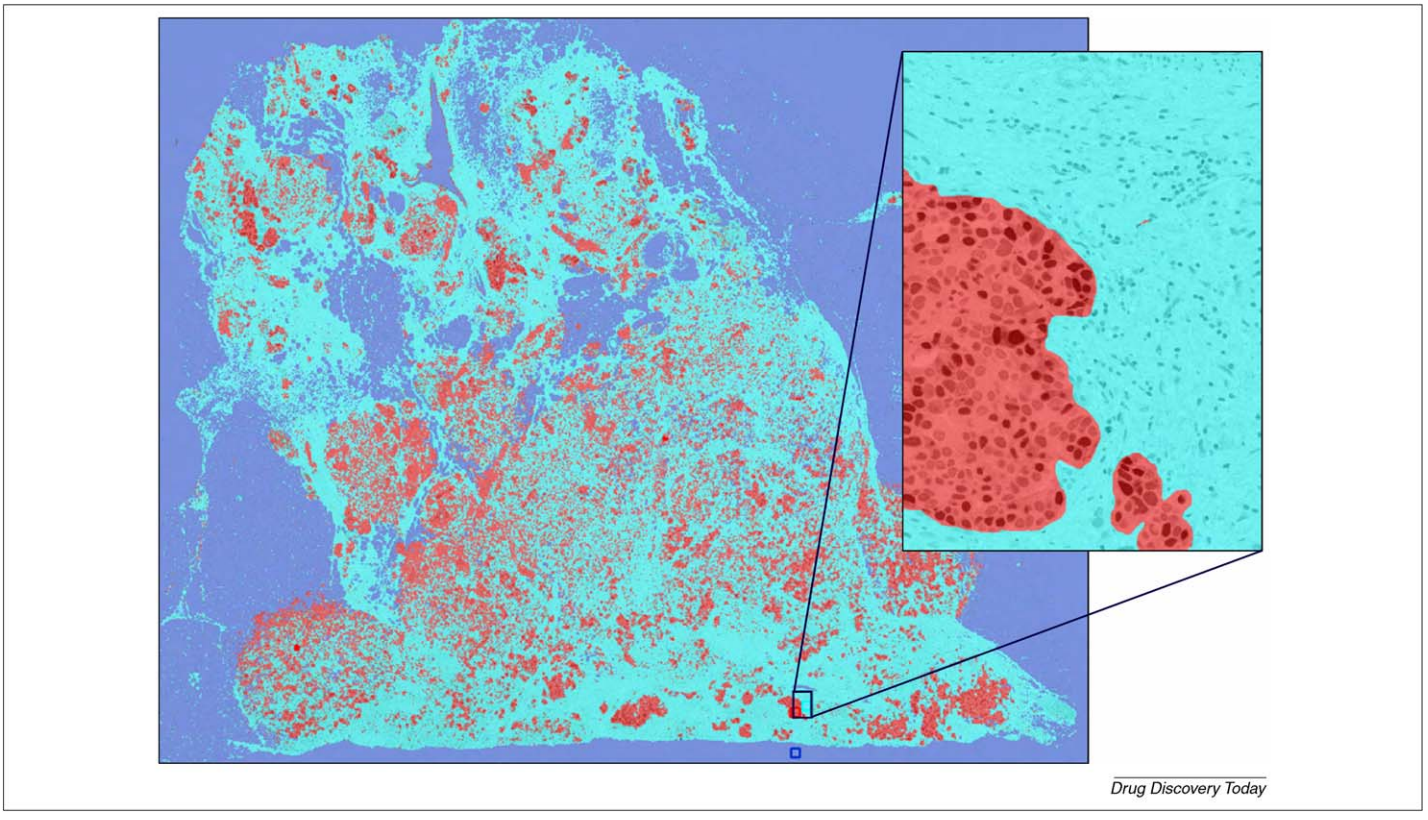

FIG. 3

With histology pattern recognition a pathologist teaches the computer to run analyses across all tissue of a similar type, greatly expanding the sample size. In the image below, histology pattern recognition has identified all the neoplastic regions in a tumor tissue sample, which will then be used to measure protein expression only on these areas.

tissue are easily recognized by pattern recognition in H&E-stained sections of mouse pancreas. It is also possible to measure alpha and beta islet cell masses through the use of double IHC staining for alpha and beta cells, in which image analysis could easily recognize both cell types, in addition to acinar cells.

Tissue-staining approaches

Image analysis can be used either on standard stains or on special stains. Standard stains, such as hematoxylin and eosin, or other histochemical stains delineate the material but are not identity specific. Image analysis approaches can be used in evaluating cellular hypertrophy or atrophy, cell numbers, tissue infiltrates (e.g. fibrosis) and other structural alterations. A good example would be quantitating cross-sectional area of fibrosis (blue stain) in tissue using a Masson's trichrome stain.

Alternatively, specific stains from protein histochemistry, IHC or *in situ* hybridization can be quantified using image analysis. However, the complexities of immune staining can lead to false negatives and false positives. False negatives can result when antibody is inappropriate, denatured or used at the wrong concentration; through a loss of antigen through poor fixation and/or diffusion; or because of the presence of antigen at a density below level of detection. Similarly, false positives can occur from several factors, including cross-reactivity of antibody with unintended antigens, nonspecific binding of the antibody to the tissue, presence of endogenous peroxidase or avidity for the avidin–biotin complex, entrapment of normal tissues by tumor cells and leakage of protein from adjacent cells with subsequent absorption by target tumor cells.[15] Despite the potential for error in staining, the biggest obstruction to effective analysis occurs when biomarkers are accurately stained but occur naturally in both target and nontarget tissues.

Image analysis results depend on proper histological processing

There are several histology guidelines that need to be followed to ensure optimal accuracy in image analysis.[16–18] First and foremost is the fixation. The longer a tissue sits unfixed at the prosector's bench, the greater the chance for degradation of the target biomarker, especially if it is protein and subject to autolytic processes. Protein-preserving fixatives such as Hepes-glutamic acid buffer-mediated organic solvent protein effect provide preservation of protein antigens for IHC. Samples should be prepared by thinly slicing the fresh tissue into small pieces, placing them individually in perforated cassettes and immersing the filled cassettes quickly into cold buffered formalin. This is because 10% buffered formalin penetrates tissue at a relatively slow rate of approximately 1 mm/hour, and thick tissue sections might not fix adequately. Insufficient fixation is frequently manifested by uneven IHC or *in situ* hybridization staining in which the edges of the sections have more intense staining than the center of the section ('edge effect'). Uneven staining of tissue sections will cause problems in image analysis. Occasionally, immediate exposure to the fixative will actually inhibit staining or inactivate the biomarker, and this will be evidenced by lack of staining at the margins of the section. If tissue samples are being fixed from a large study, it is important that all samples from all animals be handled in a uniform manner to avoid any possible bias in staining during later processing. Any possible inadvertent dose-related alterations as a result of uneven fixation should be minimized by randomization of animals for submission to necropsy. It is also important that standard operating procedures be strictly followed by all necropsy prosectors to assure that tissues are harvested in a uniform manner at necropsy. This will assure that given tissues will be removed by similar handling and be subjected to similar short periods of time before fixation.

IHC tissue samples should not be fixed for periods longer than 24 hours to avoid excessive cross-linkage of protein. Trimming of the tissues for embedding should be performed in a uniform manner such that the same plane of section is allowed for all samples in a study. All similar tissues for a study should be processed in the same batch, especially if automated processors are used. If this is not possible, it is imperative that identical conditions are documented and that there is overlap of the same tissue in different batches to enable quality control comparison of staining for the same sample.

After staining has been completed and the slides have been scanned, the digital images of the samples must be subjected to a preliminary evaluation by the image analysis tools to be used. This is to enable analysis values to be adjusted optimally for all slides in the study and will assure that varying stain intensities between slides are fully recognized by the analysis tool. After this, the final image analysis can be performed in a batch processing action across all images in a study for data collection purposes.

Anatomic consistency in sampling is mandatory in obtaining accuracy in image analysis for pathologists. Tissues have distinct regional differences in anatomical substructure and metabolism that must be taken into consideration for any type of analysis. For example, pancreatic islets vary in number depending on the region of the pancreas examined; intestinal and respiratory epithelia also vary in a regional manner, and hepatocytes differ from centrilobular to periportal regions. Despite these inconsistencies, sampling specificity will depend on the universality of the marker being measured. If a marker is known to be widespread in the liver, for example, then the sample area can be larger and could be so nonspecific as to include numbers of lobules rather than just one portion of a lobule. Such a case could lend itself more readily to random sampling procedures for statistical advantages. With regard to representative (or random) sampling, it might be easier to analyze the entire cross-section of an organ than to institute a complex and difficult random sampling procedure.

Concluding remarks

The ability to digitize entire tissue specimens on slides and subsequently perform morphometric analysis on the images is valuable in the rapid and consistent measurement of tissue features and biomarkers for pharmaceutical research and development. Image analysis of specific target tissues can be particularly challenging, such as in cases with large and morphologically intricate areas of tissue or when tissue staining is nonspecific. Histology pattern recognition is a useful preprocessing utility capable of identifying and categorizing specific histologic tissue types, thus enabling subsequent analysis of target regions by standard image analysis tools. New technologies in scanning and image analysis help to overcome the lack of access to pathology experts in discovery research and will give investigators more confidence in quantitative data concerning efficacy and toxicity.

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