JULY 2017 # 32

the Pathologist

(Bio)Banking on Pathology's Future

Pathologists must embrace their increasingly vital role as tissue archivists

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Case of the Month

Kidney Biopsy

The kidney biopsy specimen shown here was obtained from a 22-year-old woman found to have microscopic hematuria and mild proteinuria. Several serologic tests for autoimmune diseases gave positive results, including the tests for antibodies to double-stranded DNA and the Sm antigen.

What is the most likely diagnosis?

a Systemic lupus erythematosus

b Wegener granulomatosis

c Goodpasture syndrome

d Juvenile rheumatoid arthritis

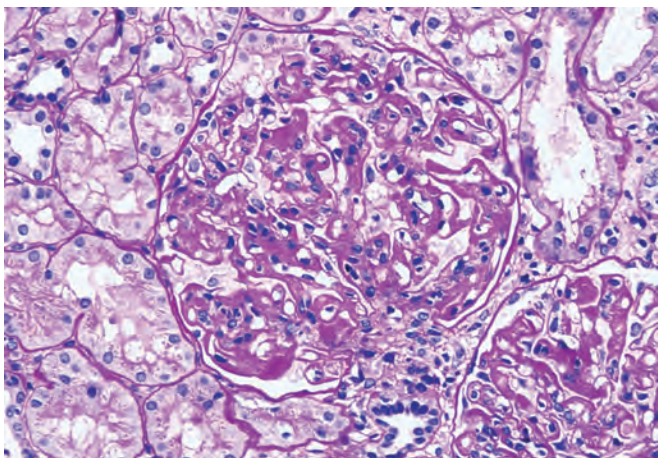
Answer to last issue's Case of the Month…

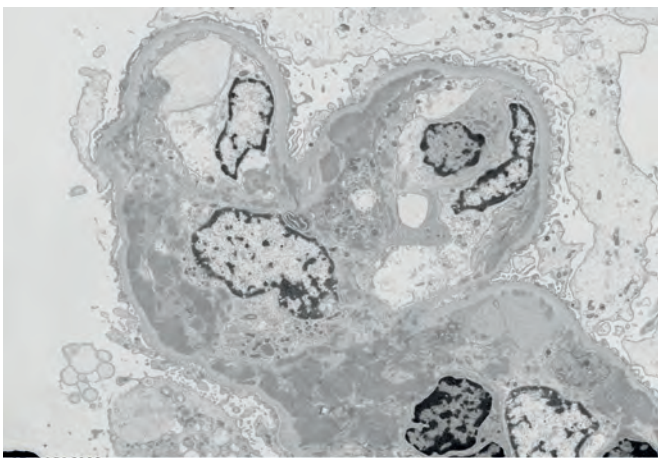
B. Clear cell carcinoma

Clear cell carcinoma of the ovary is a high-grade malignant neoplasm thought to arise from endometriosis (1). Characteristically, these tumors display several growth patterns including solid and papillary (A) and tubulocystic (B). Cells may vary from polyhedral in the solid areas to flattened in tubulocystic areas. Hobnail cells are characteristic. Tumor cell cytoplasm may range from clear to eosinophilic. Clear cell carcinomas express napsin-A and PAX8 (2). Estrogen and progesterone receptors are expressed to a variable extent, but WT1 and p53 are usually not demonstrable.

Submitted by Laura Brown, The University of Kansas School of Medicine, Kansas City, USA

References are available online at: http://tp.txp.to/0717/COTM-page





To register your guess, please go to http://tp.txp.to/0717/case-of-the-month
We will reveal the answer in next month's issue!

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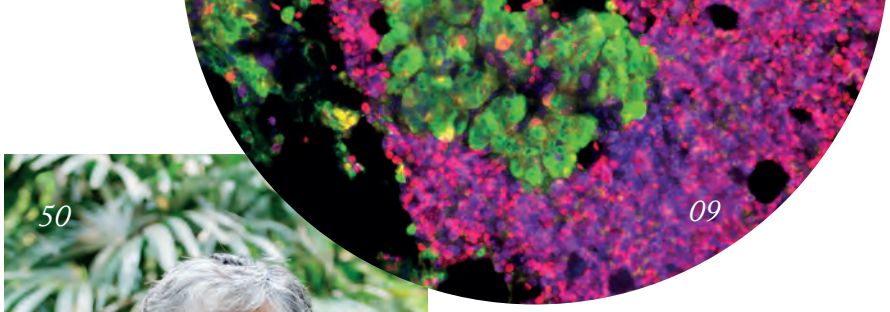
Artist's representation of biobanking, with "coins" representing samples stored in a piggybank.



Features

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Getting it Right First Time

Can pathologists and clinicians work together more closely to reduce the number of incorrect medical assessments?

"Don't worry – we'll get you back to yourself in no time..." or so I was assured by the physician after attending her clinic with a minor ailment. Several prescribed medications and as many days later, worsening symptoms encouraged me to attend a follow-up visit for a second opinion. The outcome? A different diagnosis, new medications, and an insistence that I stop taking the previously prescribed drugs. My recent experience was a only a minor case of diagnostic error, but it did get me thinking of its impact: the financial cost of two consultations and an array of incorrectly prescribed medications; the potential unnecessary side effects; my accidental contribution to the growing problem of antibiotic resistance; lost productivity...

I know that everyone in the healthcare system works to the best of their ability using the information available – and often with limited resources. But I did wonder how pathologists might help to reduce the incidence of errors that take place before a sample even gets to the lab (if, indeed, it makes it there at all). Can pathologists influence the diagnostic process at the primary care stage? And, if so, how? I would love to hear your thoughts (edit@thepathologist.com).

Last year, we published a two-part cover feature on diagnostic error (1, 2). Our experts spoke candidly about the extent of the problem in the laboratory. They deliberated over the right and wrong way to approach disclosure, discussed the legal consequences of admitting a mistake, and reflected on their responsibility to communicate directly with patients and other medical professionals. We also presented examples of successful programs designed to minimize the occurrence of error and to improve disclosure protocols. One contributor delved into the psychology of the decision-making process; according to her research, once we believe that we have identified the right cause, our minds are unlikely to be open to other possibilities.

As pathologists and lab medicine specialists, the value and expertise that you bring to the diagnostic and treatment decision-making process is immeasurable. Though many hospitals and institutes have their own systems in place to encourage regular communication between clinician and pathologist, I'm sure you'll agree that there is room for improvement. There is no one size fits all solution and no guidelines to support effective cross-discipline communication or error disclosure, so I would also be very interested in any examples of communication programs that have worked for you. Please email edit@thepathologist.com. I'm looking forward to sharing your experiences.

Fedra Pavlou
Editor

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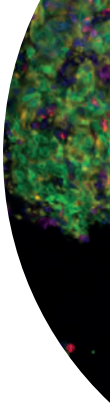
March 10, 2017.



Upfront

Reporting on research, innovations, policies and personalities that are shaping pathology today.

Do you want to share some interesting research or an issue that will impact pathology?

Email: edit@thepathologist.com

Methyl Markers

Epigenetics may predict the risk of developing anal cancer

The human papillomavirus (HPV) vaccination has significantly reduced the incidence of cervical cancer – but other HPV-positive cancers are on the rise. Anal cancer, in particular, is a problem because diagnosis is difficult and uncomfortable – both physically and psychologically. The current standard of care for diagnosis includes cytology, digital anal/rectal examination, high-resolution anoscopy, human papillomavirus testing, HPV16 genotyping, and staining of cells and biopsies for p16 and ki-67 biomarkers. Not only is this approach time-consuming and expensive (the algorithms are complex and need to be done as often as every few months), but it also allows many anal cancers to slip through. And, when they don't, overtreatment (and its serious side effects) is a problem because doctors have no way to tell which anal lesions may become cancerous.

To alleviate the burden on both patients and health care providers, Attila Lorincz and his colleagues sought an answer in epigenetics. The group's study (1) involved examining the epigenetics of anal biopsy specimens from 148 patients. They were expecting to find – if anything – a complex set of biomarkers requiring perhaps hundreds of genes. Instead, just two gene regions provided a remarkably accurate prediction of a patient's risk of lesion progression.

"We measure DNA methylation in certain regions of the HPV16 genome and in a human tumor suppressor gene, *EPB41L3*," explains Lorincz. "Methylation in these DNA regions disrupts normal cellular controls and allows anal epithelial cells to grow unchecked. With time, the tissues become malignant. The two gene regions we use were carefully selected and work very well as biomarkers because they are closely related to the carcinogenesis mechanisms in anal epithelial cells." The same gene regions are very good biomarkers of cervical and several other epithelial cancers – meaning that the mechanisms in these diseases may be the same or highly related.

At the moment, the researchers are using their biomarkers to look more closely at other important epithelial cancers, such as oropharyngeal cancer. They are also looking into how far in advance the risk of anal and cervical cancers can be predicted – but the results of these studies will take time, as they require large numbers of patients and many years of follow-up up. Says Lorincz, "In the long term, we would like to see DNA methylation tests available routinely to men and women who may be at risk of anogenital cancers." *MS*

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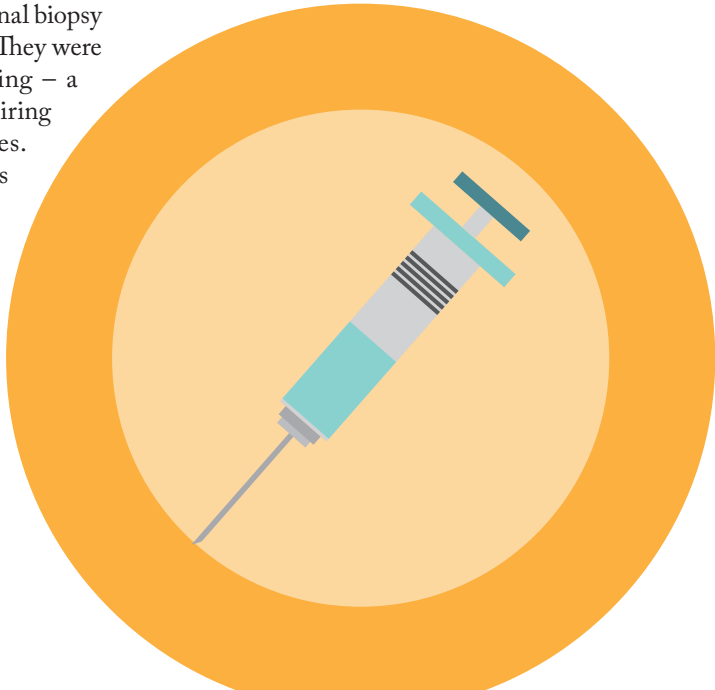

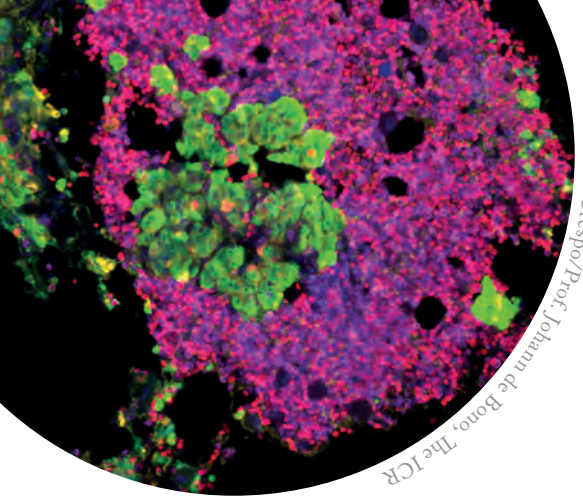

Mateus Crespo/Prof. Johann de Bono, The ICR

The Testing Trinity

A simple blood test can predict prostate cancer patients' response to treatment, monitor them during therapy, and reveal evolving tumor resistance

Although the debate over whether or not to screen asymptomatic individuals for prostate cancer rages on, not all testing is equally controversial. In men with advanced prostate cancer, for instance, better testing can improve treatment selection and follow-up care. Scientists from the Institute of Cancer Research (ICR) and the Royal Marsden NHS Foundation Trust have developed a novel, three-in-one blood test that can identify patients for therapy with PARP inhibitor drugs, detect non-responders after treatment initiation, and monitor the cancer itself for signs of evolution and treatment resistance (1).

"By looking at circulating free DNA (cfDNA), we were able to identify mutations linked to responsiveness to olaparib (predictive biomarker), identify within four to eight weeks which patients will benefit substantially (response biomarker), and pick up tumor evolution events leading to drug resistance (resistance biomarker)," explains Joaquin Mateo, Clinical Research Fellow at ICR. The most common changes Mateo and his colleagues see in castration-resistant prostate cancer are in the androgen receptor gene, *TP53*, and *ERG* – but he also points out that up to a quarter of patients exhibit defects in homologous recombination genes and may benefit from different therapies.

Who is the ideal patient for the new blood test? "We often see patients with bone metastatic disease who have already progressed to two to three lines of standard therapies," Mateo says. "The tumor may have evolved since the original biopsy, but acquiring tumor tissue repeatedly before a new therapy is challenging. In these patients, cfDNA offers us the possibility for an 'up-to-date' genomic screening of the tumor." Mateo cautions that introducing genomics into patient management is challenging, as it necessitates specialty training – but he thinks it will transform the way prostate cancer is managed. "There are already pathologists, particularly in the United States, who specialize in the use of genomics as a diagnostic or monitoring tool for cancer patients – so why not around the world, and why not in prostate cancer?"

The technology still needs to be standardized across laboratories, and the data processing must be simplified – it still requires significant bioinformatics input not normally available outside academic institutions. "This work is already ongoing," says Mateo. "And certainly, in the next five years, we will see plasma DNA becoming part of the assessment of cancer patients." He and his colleagues are running a second clinical trial, aiming to recruit up to 90 patients with mutations predisposing them to olaparib sensitivity. By the end of 2017, they hope to have completed the trial and gained a better understanding of how changes in plasma DNA reflect truly functional tumor changes. And, sooner rather than later, they hope to see the assay and analysis methods fully standardized, so that physicians everywhere will be able to use the test to improve and extend their patients' lives. *MS*

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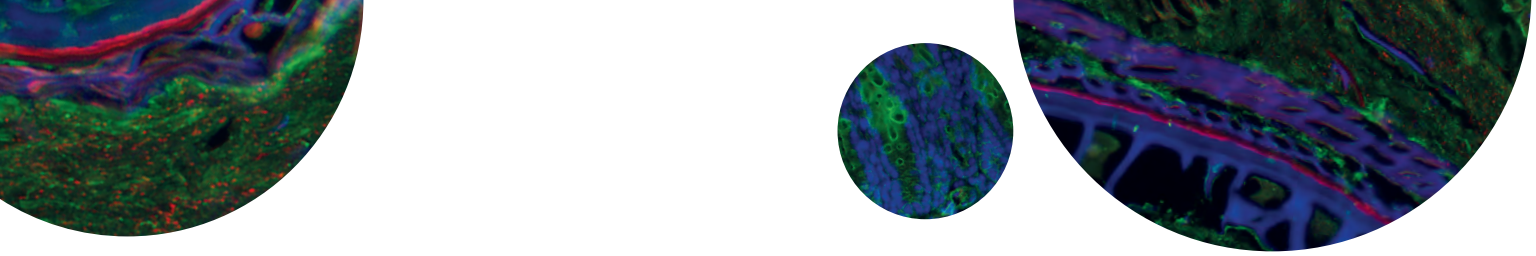

Inside Intestinal Disease

Sheena Cruickshank explains how examining gut mucus microbiota can lead to earlier diagnosis – or even prediction – of inflammatory bowel disease

Inflammatory bowel diseases (IBD) are relapsing and remitting chronic conditions of the gut that have a major impact on patients' quality of life. Current therapeutic approaches aim to reduce symptoms – but their effectiveness varies, and patients may develop tolerances to the drugs. As such, it's really important to have ways of assessing disease activity to better manage patients. Identifying microbial alterations associated with IBD could provide a diagnostic tool that enables us to spot disease earlier and minimize damage. It also contributes to better understanding of the underlying mechanisms associated with disease pathogenesis, allowing the design of improved therapeutic strategies.

The first line of defense against potential microbial invasion in the gut is a viscous layer of mucus that covers the intestinal epithelium. In our study (1), we excised the relevant mouse gut segment, opened it up longitudinally, and washed to remove residual luminal contents before scraping off the mucus for further testing. It's a difficult procedure to translate into human studies because most patients undergo routines such as colonic lavage before they are biopsied, resulting in samples that may not fully replicate the in situ bacterial communities. However, an in situ method of mucus sampling without colonic lavage that can be performed in the clinic was recently developed (2), so in the future it should be easier to get patient mucus samples. Testing would involve either qPCR or a form of protein analysis, such as ELISA.

Given that differences in microbiota composition start in the mucus before the onset of inflammation, our findings provide a framework for identifying temporal and spatial changes in the most relevant microbial communities that underpin subsequent development of IBD. Now, we are exploring whether the shifts in mucus microbial communities correlate with changed function and altered metabolite profiles. We are also developing methodologies to analyze microbial networks to develop better predictive strategies. *SC*

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Battling Blood Cancer

The FDA has authorized a new diagnostic test for hematological malignancies – and simultaneously established "special controls" for future tests

Blood cancers are complex; the symptoms are often vague and general, leading to late diagnoses – and with well over 100 different types of hematological malignancies, it's not always easy to zero in on a conclusive diagnosis. When leukemia (or a similar disease) is suspected, attempts at diagnosis are painful and invasive for the patient, and time-consuming and labor-intensive for physicians. In June 2017, the FDA authorized a new test (Beckman Coulter's ClearLLab LS) that aims to provide a simpler, more consistent way of detecting cancer-specific cell surface markers in blood, bone marrow, or lymph node samples (1).

Cell Type	*Cell surface markers detected*
T cell	CD2, CD3, CD4, CD5, CD7, CD8, CD45, CD56
B cell	Kappa, lambda, CD5, CD10, CD19, CD20, CD38, CD45
Myeloid cell	CD7, CD13, CD33, CD34, CD45

The test becomes the first FDA-authorized product that works with flow cytometry to detect and differentiate between several different types of blood malignancies, including acute and chronic leukemias, non-Hodgkin lymphoma, myeloma, myelodysplastic syndromes, and myeloproliferative neoplasms. But the test isn't the only unique aspect of the new authorization; the FDA also established new criteria for leukemia and lymphoma tests: "special controls." Special controls work alongside the existing general controls to assure the safety and effectiveness of such tests and also lay out the least burdensome regulatory pathway for others to follow when developing similar products. With these new special controls – and an existing test already pioneering their use – the blood cancer diagnostic space looks set for change. *MS*

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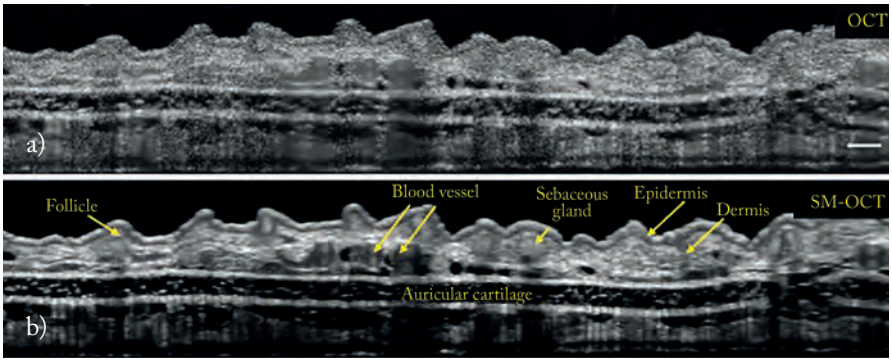
What Lies Beneath

A simple new method to decrease speckle noise could make optical coherence tomography a valuable virtual biopsy tool

Optical coherence tomography (OCT) – best known as an ophthalmology tool – is an effective way of examining basic tissue structure. The technique illuminates a tissue sample with laser beams and collects the light that bounces back, generating an image of what lies beneath. Why, then, is it not more commonly used in tissue diagnostics? Speckle noise, which results from interference between laser light waves, is an unavoidable side effect that limits OCT's diagnostic capabilities by hiding fine tissue structures. Until now, there has been no effective solution – but an optical diffuser setup may have overcome the problem (1). The study's lead author, Orly Liba, tells us more.



A mouse ear pinna visualized using a) traditional and b) speckle-modulated OCT, showing the decrease in speckle noise with the latter technique.

How can OCT help with virtual tissue biopsy?

OCT is ideal because of its ability to image tissue structure noninvasively at a very high resolution. The technique can see up to 2 mm deep inside tissue and scan in real-time, making it useful for monitoring tumor progression or response to treatment. It's also very interesting for intraoperative imaging, because it allows users to look at tissue structure without slicing and preparation. The downside to using OCT is speckle noise. Our method significantly reduces speckle noise – and, unlike other methods, doesn't degrade the effective resolution of the images.

Why is speckle noise so difficult to remove?

Speckle noise is hard to tackle because it's an inherent part of the OCT image, rather than an artifact of the imaging system. The noise can be reduced if different speckle patterns are averaged – but in static tissue, speckle doesn't change time, so there's nothing to average. Our method, speckle-modulating OCT (SM-OCT), applies local phase changes to alter the interference of light coming from different scatters within a single voxel. These changes vary speckle noise so that it can be averaged out without compromising resolution. SM-OCT was inspired by the speckle variations observed in and below blood vessels; like the diffuser in our setup, cells flowing in blood vessels introduce phase changes.

What can SM-OCT do for pathologists and laboratory medicine professionals?

OCT and SM-OCT can help by visualizing the areas of interest in large tissue samples – or even intraoperatively. The technique lets pathologists work more efficiently by identifying the abnormal or interesting areas of a given sample via SM-OCT and then obtaining tissue sections from only those regions. That allows them to slice and prepare fewer total sections, while still ensuring that no areas of interest are missed. In some diseases, a diagnosis could even be rendered entirely by SM-OCT, thereby sidestepping the need for biopsy; however, confirming that potential will require clinical trials on a use-case by use-case basis.

Is it ready for the clinic?

The move to the clinic is our next step. We can apply our method to existing commercial OCT systems with off-the-shelf components, meaning that existing OCT systems can be "upgraded" to include SM-OCT speckle reduction without significant cost. We are interested in implementing the technique for retinal imaging, skin imaging (for improved cancer diagnosis), and intraoperative imaging (for better tumor margin detection and selecting the best regions for biopsy collection).

SM-OCT can already reveal fine structures we've never previously seen via OCT – for instance, Meisner's corpuscle (the nerve bundle in human fingertip skin). Ultimately, we believe that clinical SM-OCT will combine the best qualities of our current methods: the resolution and clarity of a microscope with the efficiency and tissue preservation of OCT.

Reference

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In My View

In this opinion section, experts from across the world share a single strongly held view or key idea.

Submissions are welcome. Articles should be short, focused, personal and passionate, and may deal with any aspect of laboratory medicine. They can be up to 600 words in length and written in the first person.

Contact the editor at fedra.pavlou@texerepublishing.com

Autopsy on Autopilot

When it comes to investigating death, we must first ask ourselves, "What is the question?"

By Guy Rutty, Chief Forensic Pathologist at East Midlands Forensic Pathology Unit and Bruno Morgan, Professor of Cancer Imaging and Radiology at the University of Leicester, UK

Invasive autopsy rates vary markedly internationally, from as little as 1 percent of deaths in Japan to as much as 20 percent in England and Wales, which suggests variable medico-legal requirements to death investigation in different countries. Clearly, not all countries places the same value on the traditional autopsy.

But even in places where reliance on invasive autopsy is high, its position as the gold standard for internal examination of the body is now being challenged. Over the last three decades, we have seen developing evidence for (and increasing acceptance and adoption of) post-mortem computed tomography (PMCT) as an adjunct to – or even a replacement for – invasive autopsy.

Those who favor the traditional technique may point out that there are weaknesses in using PMCT as opposed to autopsy. Diagnostic weaknesses of PMCT include its inability of PMCT to confidently diagnose visceral injury, bruising, sepsis, pulmonary thromboembolism and more. But focusing on these alone is an oversimplification – and it misses two important points.

First, PMCT also has distinct strengths – for example, as a rapid screen for trauma, pneumothorax and hemorrhage. The combination of PMCT and invasive autopsy identifies more significant pathologies than either on its own, and should therefore be considered the gold standard for internal examination in a death investigation.

Second, death investigation is a process with multiple stages and diagnostic tools – and their use and order depends on context. For example, audit using autopsy clearly shows that many death certificates completed by doctors record an incorrect cause of death – yet, for most deaths, this process is considered adequate.

"Death investigation is a process with multiple stages and diagnostic tools – and their use and order depends on context."

We therefore suggest that, before deciding which investigative approach is best, you ask yourself: "What is the question?"

The classic questions asked in death investigation can be simplified to "where," "who," "how," and "when." In many cases, particularly traumatic deaths, the answers are known with certainty at the outset, and yet the investigation continues. Why? Often, it's to satisfy criminal court proceedings in which the investigators are fearful of subsequent criticism should they miss anything. We

must resist this! In clinical medicine, this "defensive practice" consumes increasing amounts of healthcare resources without providing benefit, and can even result in overdiagnosis and patient harm. And it's unnecessary from a legal standpoint, too; in the UK, homicide has been investigated without autopsy, using external examination only, with no adverse effect on the judicial process (despite guidelines suggesting that both autopsy and histology are required).

The defensive approach is even more mystifying when the level of evidence required for the coroner is a "balance of probabilities." The investigation in this scenario often focuses on how the deceased died and may simply fulfill the statutory obligation to provide a medical cause of death. Different attitudes to the level of evidence required for this task explain the radically different autopsy rates in Scotland (6 percent) as compared with England and Wales (20 percent).

Research has shown that a PMCT scan with targeted coronary angiography can provide the cause of death in up to 92 percent of cases of adult sudden death of natural causes (1). We also find that, for most cases of traumatic death, there is little to be gained from autopsy after a PMCT scan, particularly if enhanced with whole-body or multiphase angiography.

We are not proposing PMCT as a replacement for autopsy. Rather, we propose that PMCT become the first-line test if internal examination is required – but after consideration of the identity, scene, history, external examination and need for toxicology. PMCT may be augmented by angiography, pulmonary ventilation, and PMCT-guided histology and microbiology samples. Only if this investigation does not provide the cause of death, or answer other necessary medico-legal questions, should the investigation proceed to either a targeted or a whole-body autopsy.

In my view, this more logical approach is hampered by the current "autopsy-centric" attitude to death investigation, which neither respects the remains of the deceased nor their beliefs. PMCT is now proven to answer the questions asked in many post-mortem investigations (1,2). A small number of county councils in England and Wales have recognized the benefits of PMCT and are now developing services.

Currently, the likelihood that a death will require investigation depends on what side of the Scottish border you live on. Whether you can avoid a traditional autopsy through the use of PMCT also depends on where you live – as well as your ability to pay. Nonetheless, I believe that in the future, commentators will look back on our society – which chooses to do perform autopsies on thousands of adults and children, despite the availability of a non- or minimally invasive alternative – and say, "shame on you."

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Rebuilding the Microscope for Digital Pathology

The cost of robotic microscopy is a major barrier to the adoption of digital pathology system – but a new approach, Fourier ptychography, may offer a solution

By Guoan Zheng, Assistant Professor, Department of Biomedical Engineering, University of Connecticut, USA

Visual examination of biopsy sections by microscopy is the gold standard for cancer diagnosis. But with the improvements in digital imaging over the past decade, there has been a worldwide upsurge in attention on digital pathology – which promises to make the prediction, diagnosis, and prognosis of cancers and other diseases better, faster and cheaper than ever. In particular, digital pathology has become a popular alternative for secondary consultation with a remote specialist due to the time saved by sharing digital images instead of transferring glass slides. That time saving has turned digital pathology from a "blue-sky" approach into a promising field of diagnostic medicine. And it will only continue to grow with the advent of a new generation of pathologists trained on digital images and the emergence of artificial intelligence in medical diagnosis.

Digital pathology currently employs whole slide imaging (WSI) systems with high-resolution objective lenses to digitize histology sections. These WSI systems use high-speed mechanical scanning to generate gigapixel images

of entire histology slides. The resulting images are complete enough to provide a quick overview of an entire section, but detailed enough to provide close-up views of areas of interest and accommodate automatic image analysis. But despite these advantages, WSI systems face several challenges. For instance, their high-magnification lenses provide the resolution required to resolve structural details, but their shallow depth of field makes acquiring in-focus images of sections with uneven topography difficult. To overcome this shortfall and reduce the need to re-scan slides, WSI systems perform focus map surveying or Z-stack imaging. Unfortunately, neither of these approaches is ideal, as focus maps can only reduce (not eliminate) out-of-focus areas, and Z-stack images create large files that are hard to view, share or archive. Finally, the need for precise mechanical movements and feedback control necessitate expensive hardware – current WSI systems can cost as much as US$150,000! The cost of acquiring and maintaining such a system is a significant barrier to adoption by hospitals, clinics, and pathology groups.

Recently, a novel microscopy technique, Fourier ptychography (1–3), has been developed to acquire high-resolution, wide field-of-view images without mechanical scanning. This technique uses an LED array for sample illumination and a low-magnification lens (typically a 2X objective) for image acquisition. Each LED element on the array illuminates the sample with one incident angle; for each incident angle, the device records one low-resolution intensity image. The images are then stitched together in the Fourier domain to produce a single high-resolution picture.

Unlike conventional microscopy platforms, the final achievable resolution of Fourier ptychography does not depend on the choice of objective lens; instead, it is determined by the largest incident angle of the LED array. It has been shown that this approach can use a 2X, 0.08 numerical aperture (NA) objective lens to produce an image with 0.5 synthetic NA. What does that mean? Essentially, that it combines the field-of-view of a 2X lens with the resolution of a 20X lens. The low-magnification lens also offers an additional advantage: a broad depth-of-field that can be extended even further with reconstruction algorithms – up to 0.3 mm, at least 50 times longer than that of a conventional platform with a similar numerical aperture. A simple, robust solution to WSI's focusing problem!

Fourier ptychography is currently in its infancy, but I anticipate that it will continue to grow and expand. I look forward to seeing the new insights it brings to the development of digital pathology platforms in the future.

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Educating the Digital Generation

Adjusting to new technologies can be difficult – but rising to the challenges could change the face of medical training

By Eduardo Alcaraz Mateos, Pathologist, Department of Pathology, University Hospital Morales Meseguer, Murcia, Spain

Digital pathology has been on the scene for several years now, and we can find plenty of literature about its implementation and advantages in education – but there is a distinct lack of concrete methodologies. How exactly do we apply digital pathology to benefit students?

As with most medical professions, the theoretical aspect of pathology is mostly taught using traditional study and memorization. But with digital pathology, we can do much more. And I feel we must take advantage of everything that it has to offer our new students from the "digital generation". We should all be striving to share a vision of pathology that radiates unparalleled attractiveness – and that means integrating technology into our learning environments.

On the surface, that integration seems like a simple enough adjustment; create online tutorials or links to web content with digitized preparations to reinforce the knowledge acquired in class. But in our hospital, we wish to go further.

In 1999, Europe set new standards for medical education. The requirements change the way medicine is studied from a traditional model based on knowledge acquisition to one more focused on acquiring professional competencies (1) – skills, aptitudes and values. Alongside this change, the evaluation system is also shifting; we're seeing increasing

deployment of objective structured clinical examination (OSCE) formats. From an educational point of view, this implies reduced lecture hours in favor of supervised practical training – and that's where clinical simulation comes into play.

Simulation offers students a more realistic view of our daily work while providing them with skills of undoubted value. It also improves patient safety and quality of care by allowing learners to practice techniques in a risk-free environment. Without simulation, the first time students carry out new techniques will be on patients.

My colleagues and I have been developing clinical simulation programs in fine needle aspiration (FNA) with phantom models (WO/2016/185077 and WO/2017/109241) since 2013. We work with third-year medical students from the University of Murcia (and international exchange students through IFMSA – the International Federation of Medical Students' Associations). Each student is exposed to a clinical experience and individualized scenario that includes anamnesis, physical examination, asepsis/antisepsis measures, and finally the FNA procedure to obtain optimal material for study. A facilitating teacher accompanies the students throughout the procedure and enriches each case with digitized preparations – both cytological smears and biopsies – for an understanding of not only the procedure, but also each disease. The combination of simulation and digital facilitation ensures that each student acquires puncture skills with context.

But FNA isn't the only use for simulation. Starting in 2014 (and achieving full development two years later), we implemented simulation workshops in macroscopic dissection using handmade silicone task simulators fabricated to resemble surgical tumor resections. Students have the task of correlating the surgical specimen with the request form, carrying out measurements, and conducting the gross – that is, describing the specimen and margins, sectioning, and including in cassettes. Each sample is also accompanied by a histological correlation and final resolution of the clinical case, using whole slide imaging (WSI) in large-format monitors to understand aspects such as macroscopic management, assessment of surgical margins, microscopic visualization and diagnosis, and prognostic significance.

In my opinion, the training of medical professionals must, of course, include theoretical knowledge – but, vitally, it should also include technical skills obtained through practical experience. The importance of that hands-on education is sometimes underestimated by teaching staff, who don't or can't offer appropriate methodologies with truly useful and standardized knowledge for the student. Often, they only show what a professional is doing at a particular moment, resulting in non-uniform practical training among students and a missed opportunity to better "show off" our specialty and attract potential new pathologists.

That said, I acknowledge that the implementation of this model is difficult. It requires time, greater economic investment, more teachers, more equipment and better infrastructure. At present, the high ratio of medical students to teachers can make it hard to offer these types of time-consuming, interactive activities; unfortunately, it favors a traditional form of teaching that is increasingly insufficient for the acquisition of competencies and clinical skills. With so many different constraints to balance, simulation presents a challenge for teachers (who may never have been taught how to teach) – but also an opportunity like never before. Perhaps the ability to rise to this challenge is truly the "art of teaching."

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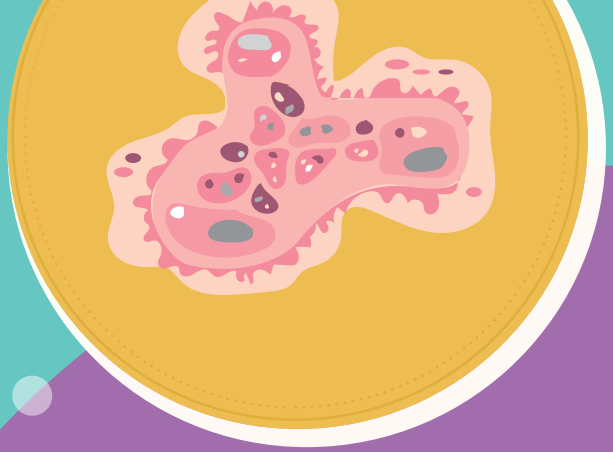
(Bio) Banking on PATHOLOGY'S Future

The field of pathology is ever-changing – but as precision medicine and molecular techniques become integral to the laboratory, pathologists need to embrace their increasingly important role as tissue archivists



Curating Pathology's Future

Biobanks are vital to biomedical research and clinical diagnostics, but we have a great deal of work to do before we can realize their true potential

By Fay Betsou

The word "biobank" first began to take off in print in the mid-2000s. More than 15 years ago, an Internet search would have returned almost nothing; today, there are over a million results. It's a very short existence for a concept that I believe is vital to modern pathology – both in research and in the clinic.

The biobanking initiative first came from the Organization for Economic Cooperation and Development (OECD), which not only started advocating for the importance of biobanks, but also insisted on the need to have an accreditation system. In the years following the proposal, the governments of various countries began funding research infrastructures for biobank operations. One such country was France, where I began my own career in the very first autonomous biobank to get ISO certification in 2005. And by 2008, the French government had developed and begun applying a national certification standard for biobanks. It's approximately equivalent to ISO 9001: a basic quality management system, but nothing more. But professional biobanks – those whose sole purpose is sample collection, processing and management – should be held to a higher standard.

The preanalytical problem

The most important aspects of a biobank are consistency and quality. When researchers come to us and say, "I need 30 lung cancer samples," we ask, "Okay, what kind of lung cancer? What kind of sample?" But most of them are not pathologists; they don't know the different histological types or sample preservation options, so they just ask us for "lung cancer." We have to educate basic and translational scientists to understand what they need in greater detail – because it's difficult to provide a professional service when the clients can't clearly articulate their requirements.

Sample characterization – clinical, pathological, immunohistochemical and preanalytical – is a large part of what we provide. Most of that may seem obvious but, until now, preanalytical characterization has been almost completely neglected despite its importance. We can't just forget to take into account the potential impact of factors such as cold ischemia time, fixative type, or even storage temperature on the downstream results; these are all critical elements that professional biobanks should track – and, fortunately, most of them do. As a result, when asked for samples, the biobank can select them according to their suitability – and the researchers can then specify in their publications where their samples originated


“THE MOST IMPORTANT ASPECTS OF A BIOBANK ARE CONSISTENCY AND QUALITY.”

and how they were handled. Without that information, it's easy to introduce invisible bias into the work – and then researchers are surprised when their findings cannot be reproduced!

Along with the Biospecimen Science Working Group at the International Society for Biological and Environmental Repositories (ISBER), we have developed a tool called SPREC – the Standard PREanalytical Code (1) – an evolving seven-element code that summarizes the nature of the sample and its history. For instance, the seven elements of a tissue sample SPREC are:

- specimen type,
- collection type,
- warm ischemia time,
- cold ischemia time,
- fixation/stabilization type,
- fixation time, and
- storage conditions.

So your specimen might carry the code *TIS-BPS-N-E-NBF-G-P*. That would make it a solid tissue specimen (TIS), collected via biopsy (BPS), with warm ischemia time not applicable (N), cold ischemia time of 30–60 minutes (E), fixed in neutral-buffered formalin (NBF) for 48–72 hours (G) and stored at room temperature in a paraffin block (P). Don't have time to code all your samples by hand? A publicly available tool, the SPRECALC, will automatically generate the codes – and there's even a second tool to convert them into barcodes for labeling.

Controlling quality

One major source of error in biobanking is poor annotation. Most clinical and pathological annotations come from medical records that lack standardized language and, on top of that, it's not uncommon for them to be transcribed inaccurately. The other significant error source is the quality of the samples themselves; either the preanalytics aren't accurately documented or quality control tests haven't been run – or both.

Almost all of our existing samples suffer from the first problem. If you went into the average biobank today and tried to annotate its samples with SPREC, 90 percent of the time, you would simply write *TIS-SRG-X-X-NBF-X-P*, because some information was never recorded. Unfortunately, there's no way to fix that; all we can do is ensure that protocols are documented going forward. But we can solve the second problem – even if you don't know how samples were collected or processed, you can still apply quality control tests to them, or to their derivatives, and use that information to stratify them into quality categories. For example, you might extract DNA, perform a multiplex PCR, and see to what extent the genetic material is still amplifiable. Of course, that brings us to a further need: the development and validation of such quality control assays

ISO Technical Committee on Biotechnology

The International Organization for Standardization (ISO) includes a technical committee, TC 276, responsible for developing standards related to biotechnology. The committee has an active working group for biobanks and resources that is currently developing a technical standard for biobanks (DIS 20387), which may eventually be used in accreditation. The standard would make traceability and quality control measures mandatory for any institution that wishes to be compliant.

DIS 20387 is currently in the inquiry stage. What still needs to be done before it becomes a formally published standard? First, national bodies will have 12 weeks to vote and comment on the draft text, including making technical changes. Then, if successful other than technical changes, the text will be updated and submitted as a final draft international standard (FDIS) and voted on again – this time without the option of technical changes. Finally, if approved, the text will be sent to the ISO Central Secretariat for publication as the International Standard.

– but, in my opinion, that is the only solution that can allow us to use with confidence the millions of legacy samples stored in biobanks and pathology labs around the world (2).

Teaching and training

We are constantly involved in spreading the word about biobanking – why it's necessary, who can benefit, how it's done... When I worked at a university hospital in France, we organized training for clinician-researchers; now that I'm in Luxembourg, the work continues. We have developed a university certificate on biobanking that is targeted more at biobankers themselves, but we often see researchers and clinicians signing up because they want to learn more. We organize seminars at hospitals and research institutes to educate the faculty, and they are always very surprised when we explain to them, "You ask us for lung tissue – did you know that there are different histological types? Did you know that a sample with 10 percent tumor content will give you completely different results in your analyses than one with 80 percent tumor content?" It's a revelation to them. Clearly, there's a lot of work to be done!

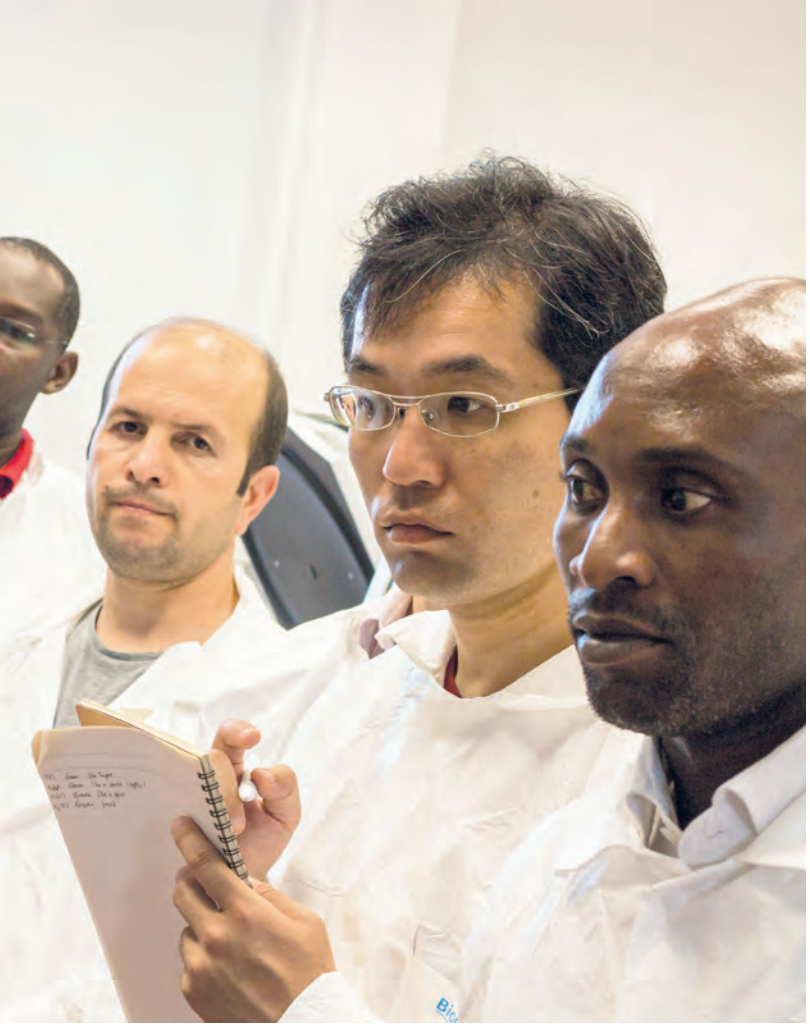
For many years, I have been saying that professional biobankers need to submit abstracts to scientific society meetings. After all, our work is applicable to every area of biomedical science: immunology, cardiology, oncology, infectious diseases, hematology, and the list goes on! So any biobanker can assemble an abstract that addresses a few key questions:

- What are biobanks?
- What kind of work do they do?
- Why are they important?
- How can they help with your field of study?

We don't do nearly enough of this kind of outreach work. In my opinion, we should be at all of the major scientific meetings. We need to make the research community aware of our services and help them to understand why they need us – and we need them.

Enabling access

The biggest obstacle to bringing researchers and biobanks together is the question of supply versus demand. If you are a researcher who needs samples and associated data and you try to request them from a biobank, you will almost never find what you are looking for. Why? Because the needs of each research project are so specific that often, even big biobanks won't have what you need. In fact, this is a subject of much discussion in the biobanking community: what is the best way to operate? Should we operate on stock and try to build a huge library of samples so that we can provide as many different options as possible? Or should we operate on project-based demand? At the moment, most



biobanks follow the first model – but experience shows that it is neither the best nor the most efficient method. Much of the time, researchers don't have a use for what we have in stock, whereas we cannot provide them with what they do need.

I think the best approach is to switch to prospective, project-driven collections – but of course, for this you need professional biobanks with all of the necessary infrastructure in place to begin collecting immediately. If you have to wait a year while you assemble an ethics committee and establish everything you need from an administrative point of view, your clients will go elsewhere – or won't be able to conduct their research at all. Professional biobanks already have the administrative and the quality management systems required. You send them your request; they begin collecting in a consistent and controlled manner; and after only a few months, they deliver exactly what you need.

I don't know of any biobanks that currently work to this model, but it is something we are trying to develop. The first step is networking. You need to be in small, bottom-up networks to provide samples efficiently; if you don't have what a client needs, it's possible that another biobank does, which prevents the need to start from scratch. This kind of functional networking already exists in a few countries – in Spain and the United States, for example, and there's a government initiative to establish something in Germany as well – but it's lacking in most places. Even in those that claim to have such networks, it's often more like a list or catalog of existing biobanks, rather than a true relationship between them.

At IBBL, we believe in "trusted biobank networks" – but it will take time to build them. In the interim, we advise potential users to locate biobanks that are serious and professional to help them get the samples they need.

It's clear that our work as professional biobankers is just beginning – not only in our sample procurement and preservation work itself, but also as documenters, educators and promoters. The future of pathology lies in biobanking, and it's up to us to step forward and make these services the best they can be.

Fay Betsou is Associate Professor at the University of Luxembourg and Chief Scientific Officer at the Integrated Biobank of Luxembourg.

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The Promise of Precision Pathology

No one is better placed than pathologists to drive the precision medicine of the future – and a new kind of pathology will be crucial

By Michael H.A. Roehrl

Precision healthcare is the future – of that, I have no doubt. But how do we go about successfully developing it for the patients who need it? The key, in my opinion, lies in the comprehensive availability of high-quality human samples for all aspects of research – from basic bench work to clinical trials. And who better to ensure that availability than pathologists? Pathology is the central specialty of personalized precision medicine. It is pathology that provides the skills, infrastructure, and scientific vision we need to lead the way in science-driven biobanking, and it is pathology that can help to ensure optimal research use of human samples. And that's why my pathology colleagues and I have taken on the task of setting up a major new initiative at Memorial Sloan Kettering Cancer Center – the Precision Pathology Biobanking Center (PPBC).

Founded in 2015, the PPBC represents an institution-spanning collaborative research center that is being built around five highly interconnected pillars (see Figure 1): next generation, "future-proof" biobanking; "big data" computer science and database development; a hub for developing and evaluating the next wave of theranostic pathology technologies (like proteomics, metabolomics, and molecular imaging); a hub for pathology to take on a proactive role in the latest generation of specimen-driven clinical trials and drug development; and a platform for pathology to develop strong joint research, development and commercialization partnerships with the private sector. It's easy to see how a thoroughly annotated, high-quality biobank underpins every one of these pillars.

Building a better biobank

When we designed the PPBC's specimen acquisition, preservation, storage, and distribution workflows, the concept of "future-proofing" was front and center: all samples (tissues, bloods, other liquids) are procured at high speed (ideally directly in the operating rooms or interventional radiology suites) and uniformly held in vapor-phase liquid nitrogen, rather than dry ice or -80˚C freezers. Previous research has convincingly shown that some of the most interesting components of the pathophysiome – like RNA, post-translational modifications of proteins, or small metabolites – degrade unpredictably, even at -80˚C, over time spans of months to a few years. In vapor-phase liquid nitrogen (which cools to below -160˚C), on the other hand, they remain stable – thermodynamics is one's friend. The PPBC banks specimens from approximately 7,000 new cancer patients per year, including surgical resections, interventional radiology biopsies, and companion blood and body fluid collections – so we certainly don't want to lose those samples just a few years down the road.

How do we prepare our samples? Lesional and matched normal tissues are flash-frozen in liquid nitrogen without further additives; then, we prepare spatially indexed formalin-fixed and paraffin-embedded (FFPE) blocks that match each sampling location of a corresponding frozen vial. Blood (frequently both pre- and post-treatment) is processed into frozen serum, plasma (double-centrifuged for use as a source of circulating free DNA), and buffy coat (white blood cell) aliquots. Of the more than 30,000 specimen units we create annually, over 1,600 units of frozen samples and 1,000 units of FFPE material are used for immediate research. The rest of the material isn't simply relegated to long-term storage, because we have many innovative projects underway. For instance, a significant and rapidly growing portion of the PPBC's activities (amounting to about 1,700 units of fresh samples) is related to "living" biobanking – the creation of organoid cultures (see Figure 2), mouse xenografts, primary cell lines, and so on.

Our biobank division has developed innovative QA/QI metrics and processes, including RNA integrity monitoring in sentinel samples and participation in international proficiency testing schemes, such as the International Society for Biological and Environmental Repositories' Integrated Biobank of Luxembourg

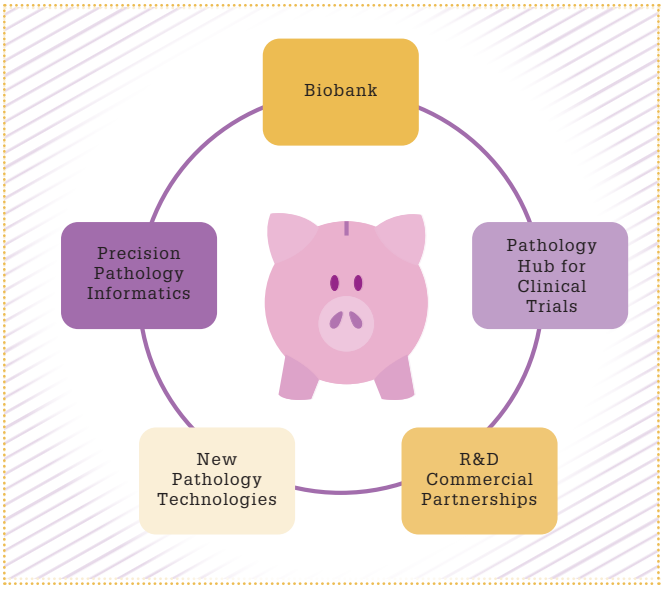

Figure 1. The five pillars of activity around which the Precision Pathology Biobanking Center is designed.

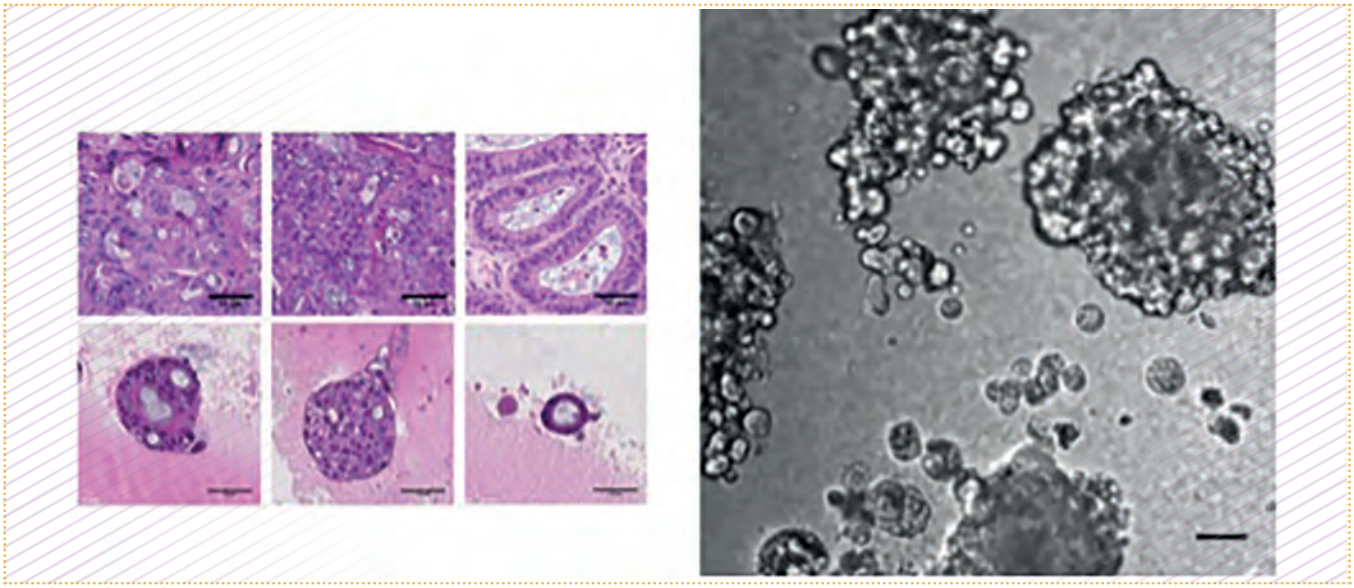

Figure 2. Examples of a "living biobank" (organoids of pancreatic adenocarcinoma). Living biobanks are an area of rapid growth, but need further innovations in biospecimen handling and preservation.

program (see "Curating Pathology's Future" on page 17). Most importantly, we made a strategic decision early on to embed our research biobanking activities intimately into existing clinical workflows. One good example is our rapid tissue acquisition setup, which takes samples from the point of acquisition to liquid nitrogen storage in less than 15 minutes. We accomplish that by pairing up licensed pathology assistants (PAs) with biobank technicians according to daily schedules and making sure that the clinical PAs assigned to biobank service on any given day aren't distracted by clinical responsibilities on those days, letting them dedicate their time and effort fully to research biobanking.

Informatics impact

A physical repository of biospecimens is only as good as the level of annotation and knowledge that can be associated with each and every specimen in the bank. Recognizing that data federation (the aggregation of disparate data sources), research databases, and smart "big data" query tools remain a major challenge, the PPBC has started to put significant effort into developing innovative data informatics and computer science tools (see Figure 3). We feel strongly that pathology as a discipline will increasingly evolve into the medical specialty of dynamic data management and big data integration to drive patient care – theranostics – rather than the status quo of "just" providing a static diagnosis.

Translated to biobanking, it means we need to build tools that cross-reference physical samples in real time to all other data we may have on a patient (clinical status, therapeutic status, imaging results, clinical trial participation, molecular features of the disease, and any other relevant information). We attempt to build a longitudinal representation of every patient, from diagnosis through stages of treatment and recurrence to long-term follow-up. We map each physical sample onto a common timeline along with all other observational or interventional medical events. For example, we could ask, "How many frozen research samples containing cancerous tissue does the bank hold from patients born after 1960 with a diagnosis of KRAS-mutated colon cancer (see Figure 4)?" As convoluted as that sounds, we can readily build much more complex Boolean queries on the fly and still have results within seconds. And it's not just to show off the power of our data organization. Queries like that one have already become instrumental tools for feasibility arguments in grant submissions and hypothesis generation for numerous biomarker studies – and we foresee even greater possibilities for them in the future.

Technology marches on

A pathology-controlled biobank is a major scientific asset for our discipline. We are currently at the beginning of a wave of disruptive technologies that I predict will become essential in our diagnostic and theranostic toolsets. With next generation sequencing reaching technological maturity in clinical laboratories, we already see new technologies (such as mass spectrometry-based deep proteomics, functional assessment of pathway activities, metabolomics, highly multiplexed immunofluorescence, ex vivo living models of drug response,

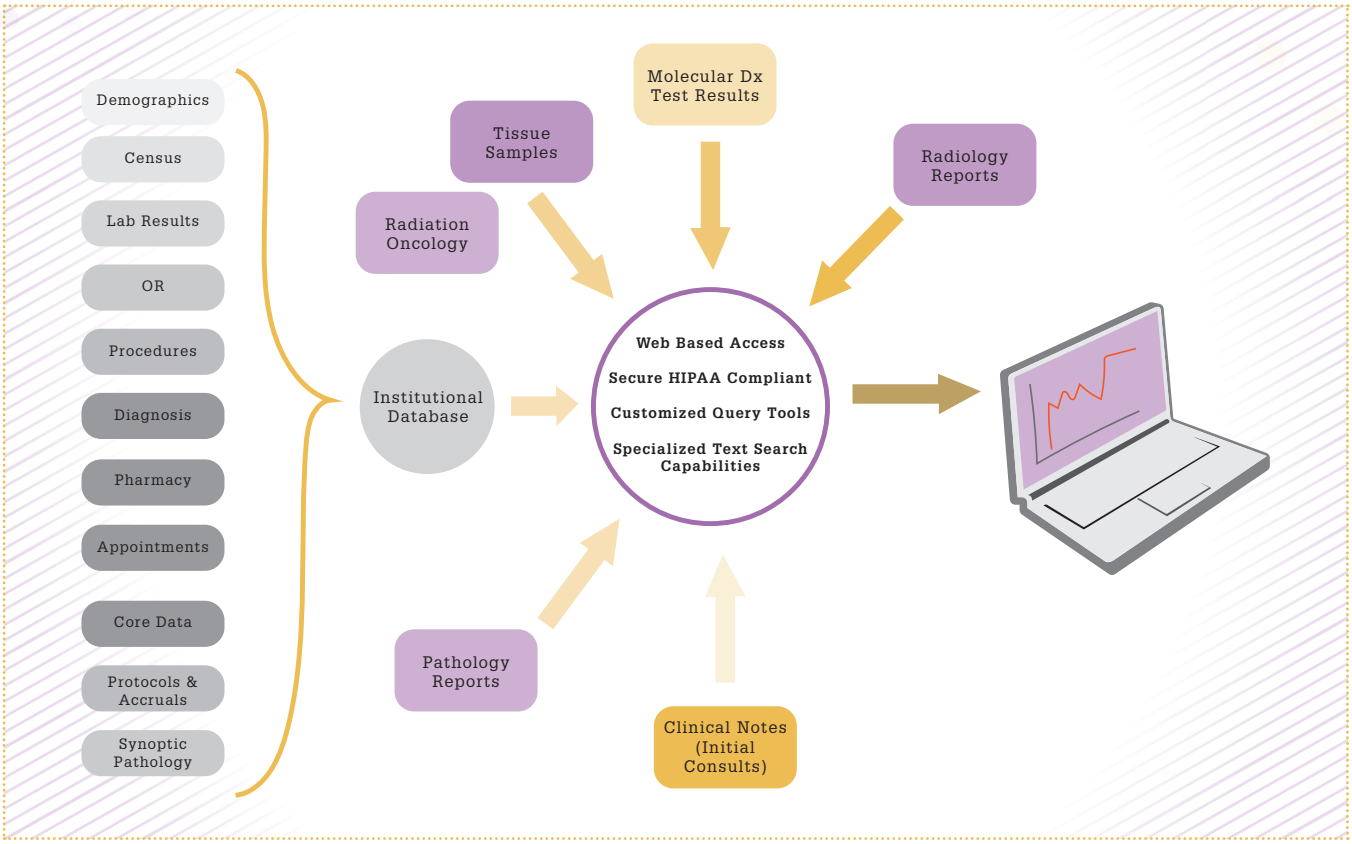

Figure 3. The organizational framework we use for research data at Memorial Sloan Kettering Cancer Center.

and more) that promise to change the way we will assess and monitor disease.

By tightly integrating biobanking into the PPBC's overall mission, the real-life evaluation and clinical assessment of new technologies becomes a natural fit. At the moment, we are assessing high-resolution Orbitrap liquid chromatography-mass spectrometry (LC-MS) as a highly quantitative, highly multiplexed tool that can precisely measure several thousand proteins in tissue in parallel. If it works the way we hope, it will be able to complement – if not replace – conventional immunohistochemistry. And not only does mass spectrometry require no antibodies, but it can also directly detect mutations at protein level and post-translational activation states, such as phosphorylation. So why are we the perfect testing ground for such innovations? Most new technologies require living and biobanked samples of the highest quality. Conventional FFPE-based clinical archives are either suboptimal or altogether unusable for these applications. Cutting-edge, forward-looking and science-driven biobanking is clearly the way forward.

Trying out trials

Pathology has not historically been a driver discipline in clinical trials or drug development, with its role often limited to providing slide review for patient enrollment or sending FFPE material to third-party trial sponsors. In the era of what I like to call "specimen-centered, molecularly driven" clinical trials (for instance, basket trials like NCI-MATCH), pathology's role is rapidly changing and our discipline is becoming a central player. This development has significant ramifications for pathology training and education, as well as for our understanding of pathology as an increasingly clinical discipline.

The PPBC has a division that provides a dedicated platform for pathology's representation at every stage of a new clinical trial; it includes design, protocol writing, budgeting, direct discussions with sponsoring pharmaceutical companies, specimen acquisition, companion diagnostic development, and any other aspect you can imagine. To provide just one example, we've created a dedicated Phase I biobank for patients on first-in-man clinical trials that provides an unmatched resource for research. We believe pathology belongs at the forefront of new medical science, and we're pulling out all the stops to make sure it gets there.

"WE BELIEVE PATHOLOGY BELONGS AT THE FOREFRONT OF NEW MEDICAL SCIENCE, AND WE'RE PULLING OUT ALL THE STOPS TO MAKE SURE IT GETS THERE."

Figure 4. An ad hoc database query using data federation between various databases (in this case research biobank, cancer registry, molecular diagnostics, and anatomic pathology).

PPBC R&D partnerships

The combination of comprehensive biobanking and new technologies provides a natural, externally visible infrastructure that now allows the PPBC – and pathology as a discipline – to engage directly with the biotechnology and pharma sector. We are enabling pathologists and commercial entities to carry out joint projects, such as co-development of new companion diagnostics, evaluation of biomarkers, or the use of new instrumentation. Such projects frequently hold opportunities for intellectual property generation. And there are even more tangible benefits; research biobanking is often difficult to support through traditional funding mechanisms, so funding raised through research and commercialization can represent a major contribution to its long-term sustainability.

We're at an exciting junction in pathology's growth as a medical specialty, and I'd say it's becoming clear that pathology-driven biobanking is both central to our core expertise and, even more importantly, a powerful enabler for many of the most promising growth areas of our discipline: precision healthcare, clinical trials and drug development, theranostics, and functional assessment and monitoring of disease. I'm eager to expand biobanking's role in pathology, and eager to see where this new platform can take our discipline next.

Michael H.A. Roehrl is a practicing pathologist, physician-scientist and principal investigator, and Director of the Precision Pathology Biobanking Center at Memorial Sloan Kettering Cancer Center, New York, USA.

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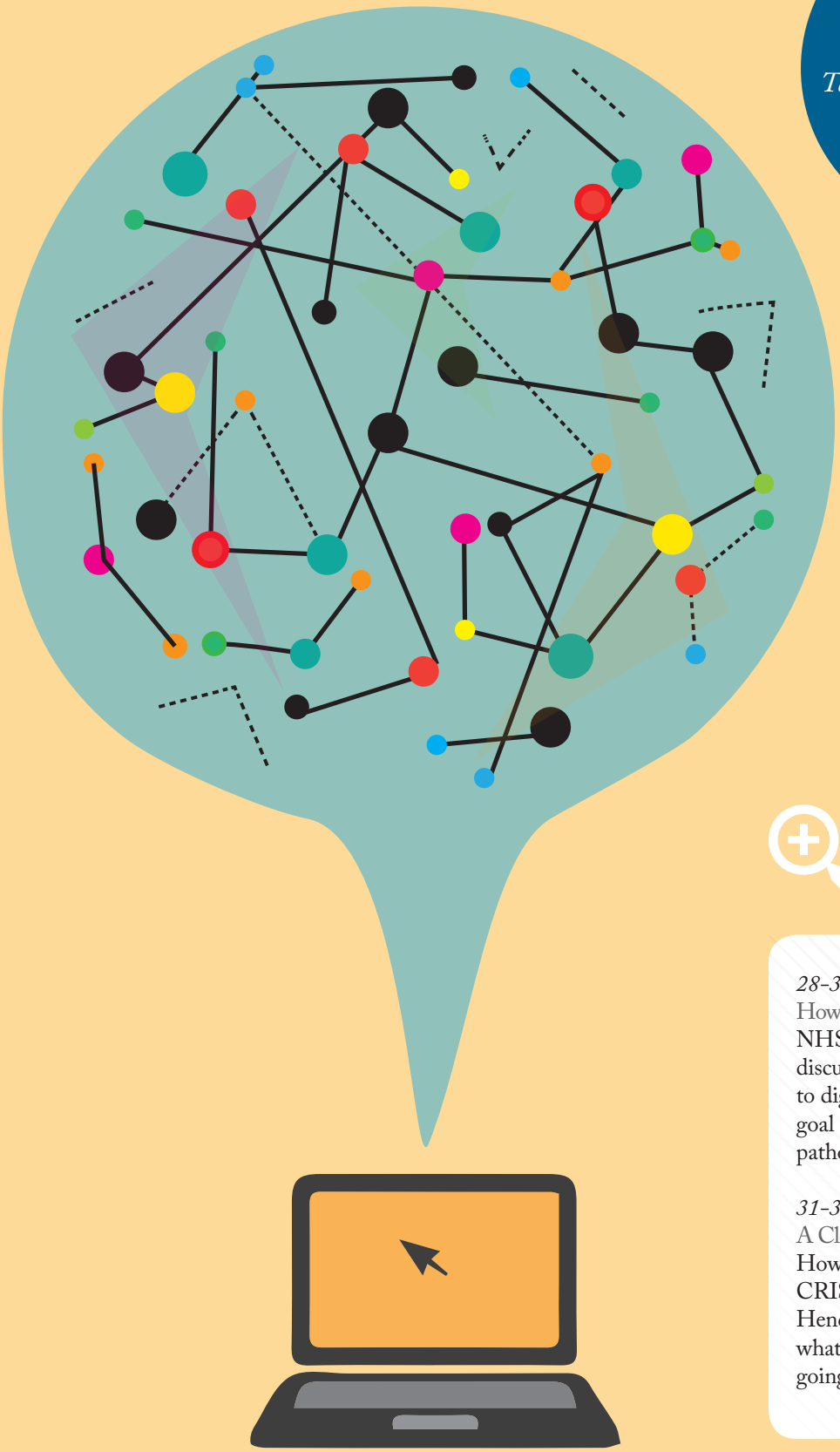
In Practice

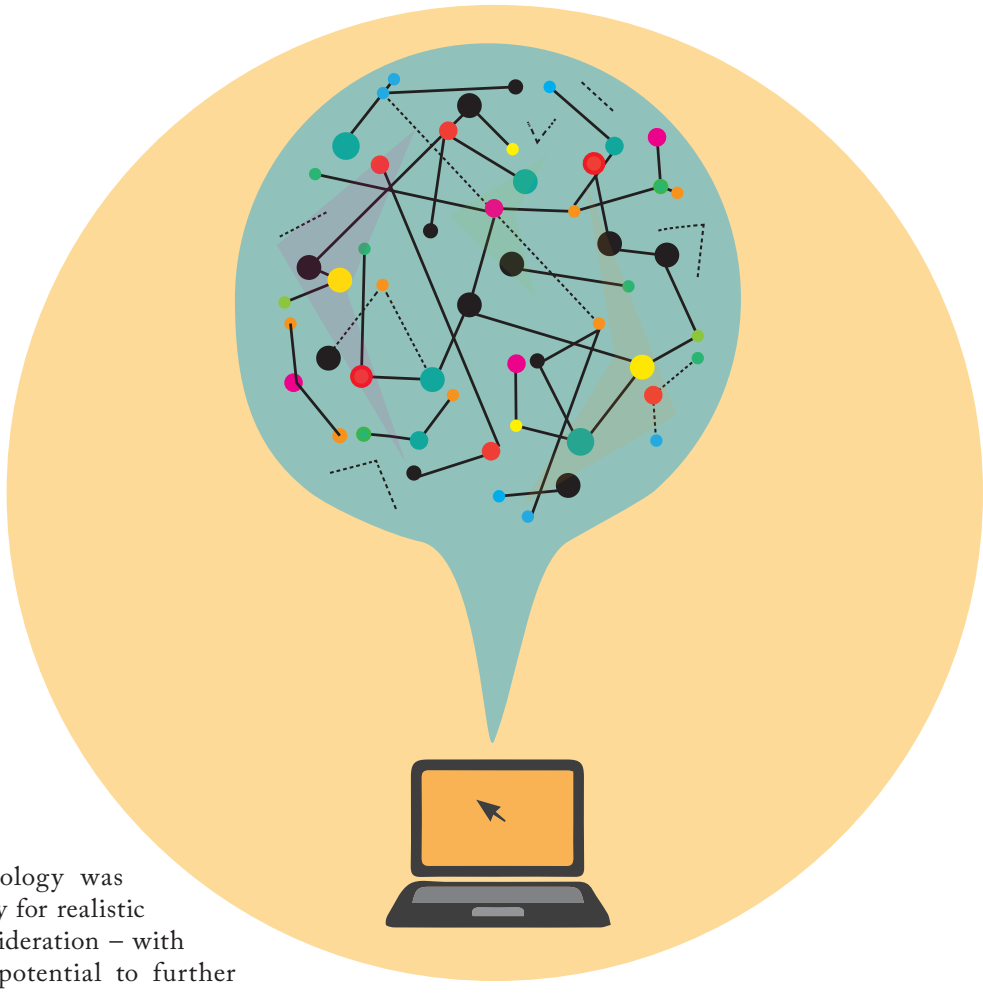
Technologies and techniques
Quality and compliance
Workflow



How We Are Going Digital

NHS Wales Health Collaborative shares the story of Wales' ongoing transition to digital pathology, the verification program used to inform the move – and the positive results seen so far

By Melanie Barker and Jane Fitzpatrick



Cellular pathology services in Wales were under pressure, with a national shortage of consultants, difficulties in recruitment, and an ever-increasing workload all adding to a recognized service sustainability risk. Rapidly advancing technology offered a potential solution to the problem in the form of digital technology. Could this technology be implemented nationally?

In the past, the very idea of going digital would have been met with a healthy dose of skepticism, but because digitization of glass slide preparations had reached a sufficient level of quality, efficiency and effectiveness, we believed digital pathology was ready for realistic consideration – with the potential to further integrate the software interface with the Wales Laboratory Information Management System (WLIMS), which would allow us to report cases within an all-digital environment. This combination of factors encouraged us to begin investigating digital pathology's potential for NHS Wales Diagnostic Cellular Pathology services.

An all-digital cellular pathology service is attractive for a number of reasons. First, it eliminates many of the time-consuming steps involved in physically transporting microscope slides to consultant cellular pathologists locally, externally, and for multidisciplinary team (MDT) review. Second, case reviews and external expert opinions can be performed electronically – and in real time – increasing access to additional diagnostic expertise and thus precision. Third, remote consultant MDT attendance through video conferencing is improved by the inclusion of images, resulting in the potential for greater subspecialization and shared working across NHS Wales.

At a Glance

- *Six Welsh health boards collaborated on a verification program for digital pathology equipment to be rolled out across the country*
- *Verification involved comparing digital reporting against traditional glass slide methodology*
- *Results were favorable, with 95 percent concordance between digital and glass slide reports*
- *Based on those results, the program is now entering its second phase: a nationwide implementation of digital technology*

One step at a time

Following the 2013 modernization of the Cellular Pathology service in North Wales – which centralized three cellular pathology services onto one site – Betsi Cadwaladr University Health Board (BHUCB) successfully bid for capital to procure a digital cellular pathology service, which was installed in March 2015. However, medical staffing and other service constraints prevented the necessary verification process for clinical use. More resources were needed, so another successful bid was submitted to the Welsh Government in November of 2015,

on behalf of the National Pathology Operational Managers Group, to fund a national program. Our plan? Two phases: verification, followed by national implementation.

In January of 2016, we agreed membership of the Efficiency through Technology Fund Digital Cellular Pathology Programme Board, drafted a program timeline, and held initial discussions with procurement colleagues. A month later, we held the inaugural Programme Board meeting and circulated a list of nationally agreed items for procurement of clinical workstations to health boards for purchase, we also identified Consultant Histopathologists participating in the verification exercise for each health board and a project team at Betsi Cadwaladr. Finally, we were ready for the verification exercise, which officially began in April 2016.

The good and the bad

The positives of the verification were that staff communicated well, used national procedures and protocols provided by BCUHB, and were enthusiastic about the verification program.

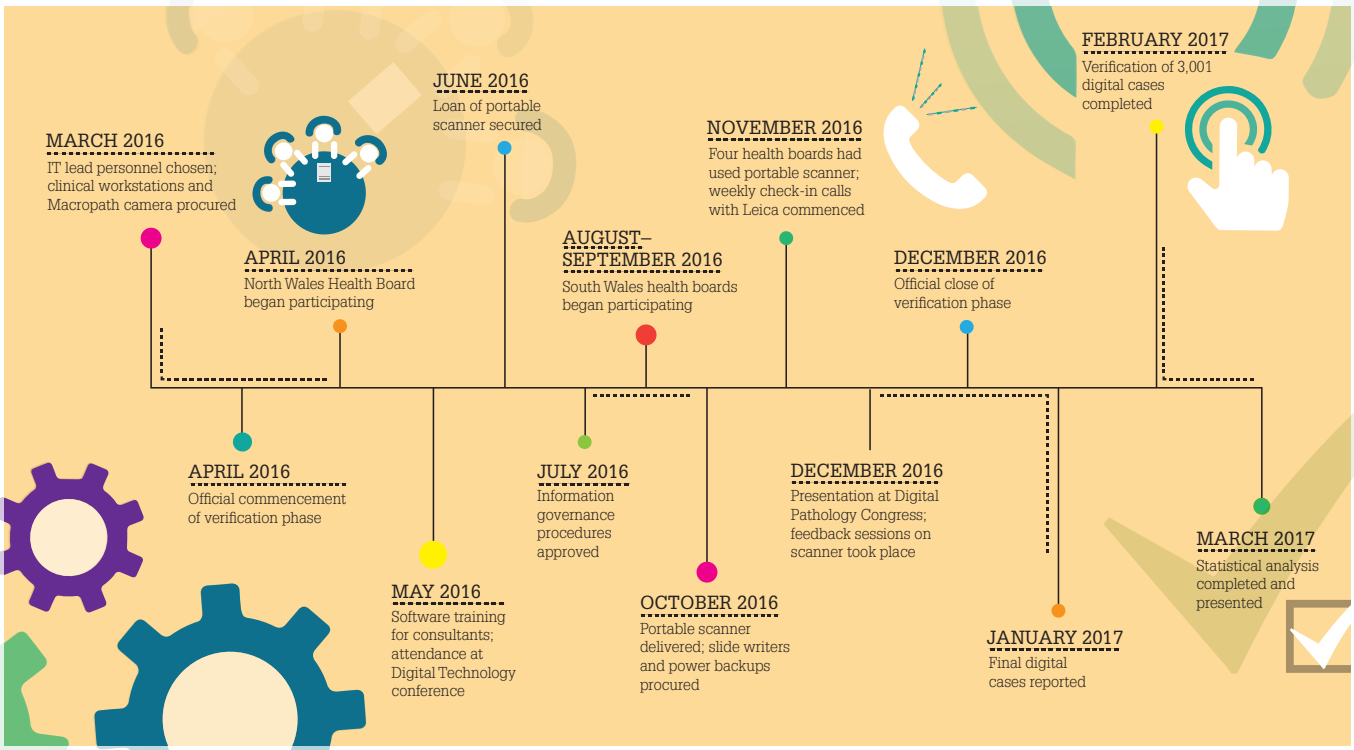
The negatives were system issues that could not be quickly resolved because of differences in local health board IT policies that resulted in delays to the verification process and frustrations in colleagues. Some of these issues and frustrations were alleviated by implementing a weekly audio conference with colleagues at Leica – our equipment supplier – which helped to resolve some of the system issues.

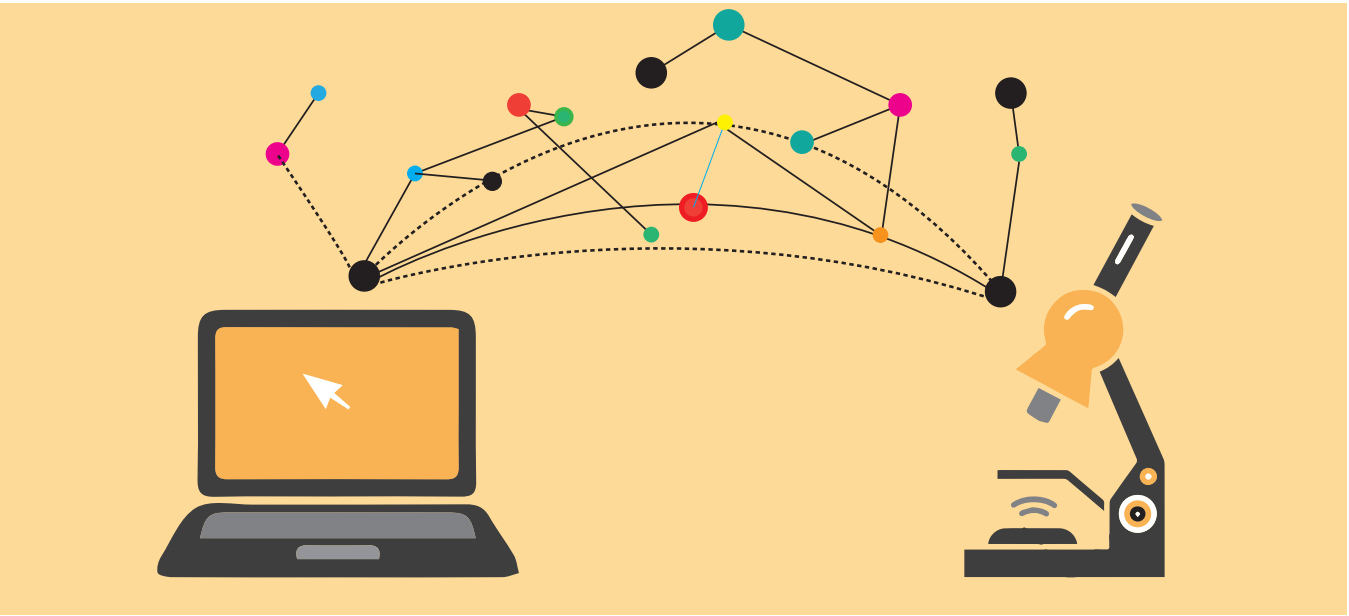
The changes in working practices experienced were handled well by colleagues which in part were due to robust procedures provided by experienced colleagues in BCUHB and an overall enthusiasm to ensure that the verification phase reached completion.

Just over a year after the verification stage began, the final statistical analysis was presented to the Programme Board, the Meeting of Welsh Histopathologists, and the Welsh Scientific Advisory Group Symposium. Our results were very promising when compared with other international studies of digital pathology, particularly in terms of concordance and accuracy. The Programme Board agreed to recommend to the NHS Collaborative Executive Group to proceed with a full rollout of digital technology – and the Chief Executives agreed to move to the second phase of the program: implementation.

Lessons learned

During the course of the verification phase, we learned a few valuable lessons that we'd like to share with other pathology services looking to make the same move.





- Information governance protocols are vital – establish them early on in the program as they are essential for sharing digital images!
- A national information technology lead would have been helpful. Although we identified local IT leads early on in the program, we did not select a national lead for the verification phase – and we think this added to difficulties we experienced with conflicting work practices between local health boards' IT departments. For the implementation phase of the program, we will be including a national IT lead as an essential role.
- Discrepancy meetings between colleagues in North and South Wales were identified as a key part of the program at an early stage. These discrepancy meetings were held between colleagues if there was a difference between results reported on glass slides and those reported using digital image. The importance of these meetings has been highlighted by clinical colleagues to discuss the variance in results, reach concordance, and additionally as a valuable peer review. We recommend that anyone undertaking a similar transition to digital implement these meetings as well.

The way forward

Cellular pathology services across South and West Wales are currently being reviewed through a separate process; the outcome of which is likely to be a service model similar to that of North Wales – namely, a reduced number of centralized sites providing service in the future. We still need to further consider the impact of digital technology on that service model, but we expect approximately the same number of slides generated, so scanner capacity should not be significantly affected. The main impact of the technology is most likely be increased flexibility for pathologist reporting – a benefit by any measure!

Given the possibility of a centralized service model for South and West Wales, part of our implementation phase involves procuring a digital pathology solution for those regions based on their activity and medical workforce profiles. The tender specification for the Betsi Cadwaladr system would serve as the initial basis for this procurement. Additionally, we can use the shared learning and national verification work from the North Wales project to inform the implementation of the South and West Wales system, meaning that the system could be deployed immediately after procurement. Within three years, the entire NHS Wales Cellular Pathology service could be entirely digital – and given the success of our initial verification phase and the benefits we've seen thus far, we're looking forward to full implementation.

Melanie Barker is Senior Programme Manager with the NHS Wales Health Collaborative.

Jane Fitzpatrick is Director of Strategic Programmes with the NHS Wales Health Collaborative and Senior Responsible Officer for the Efficiency through Technology fund Digital Cellular Pathology Programme.

A Clean (Gene) Break

CRISPR/Cas gene editing is a powerful, ever-evolving tool – but it seems to be here to stay...

Michael Schubert interviews Eric A. Hendrickson

Precise genome engineering isn't new – neither the idea of it, nor its execution. The concept of genome engineering has been around for a long time. Humans have always thought that it would be fanciful if you could go into the inner workings of an organism to make it do – or even be – something different. The ability to actually do that in lower organisms has also been around for quite a long time; in fact, the reason yeast is so commonly used as an experimental model system is because of a 1980s research breakthrough when we figured out how to replace its genes. Essentially, technology was developed to change the yeast genome to anything we wanted! Importantly, during the course of that work, we also learned that if you created a double-stranded break in the chromosome, it greatly augmented the gene editing process – a seminal observation that reverberates all the way through to our newest and best technology: CRISPR/Cas.

But after this success with yeast, researchers tried to do similar things with higher organisms and discovered that it didn't work nearly as well in most other eukaryotic cells. So for many years, people just threw pieces of DNA into mammalian cell lines and developed esoteric selections, trying over and over again to achieve practical gene editing. They only started to see success when they began applying the same double-stranded break trick they'd used in yeast. And that is, at the end of the day, the critical contribution of CRISPR/Cas – that it allows you to make a break wherever you want in the human genome.

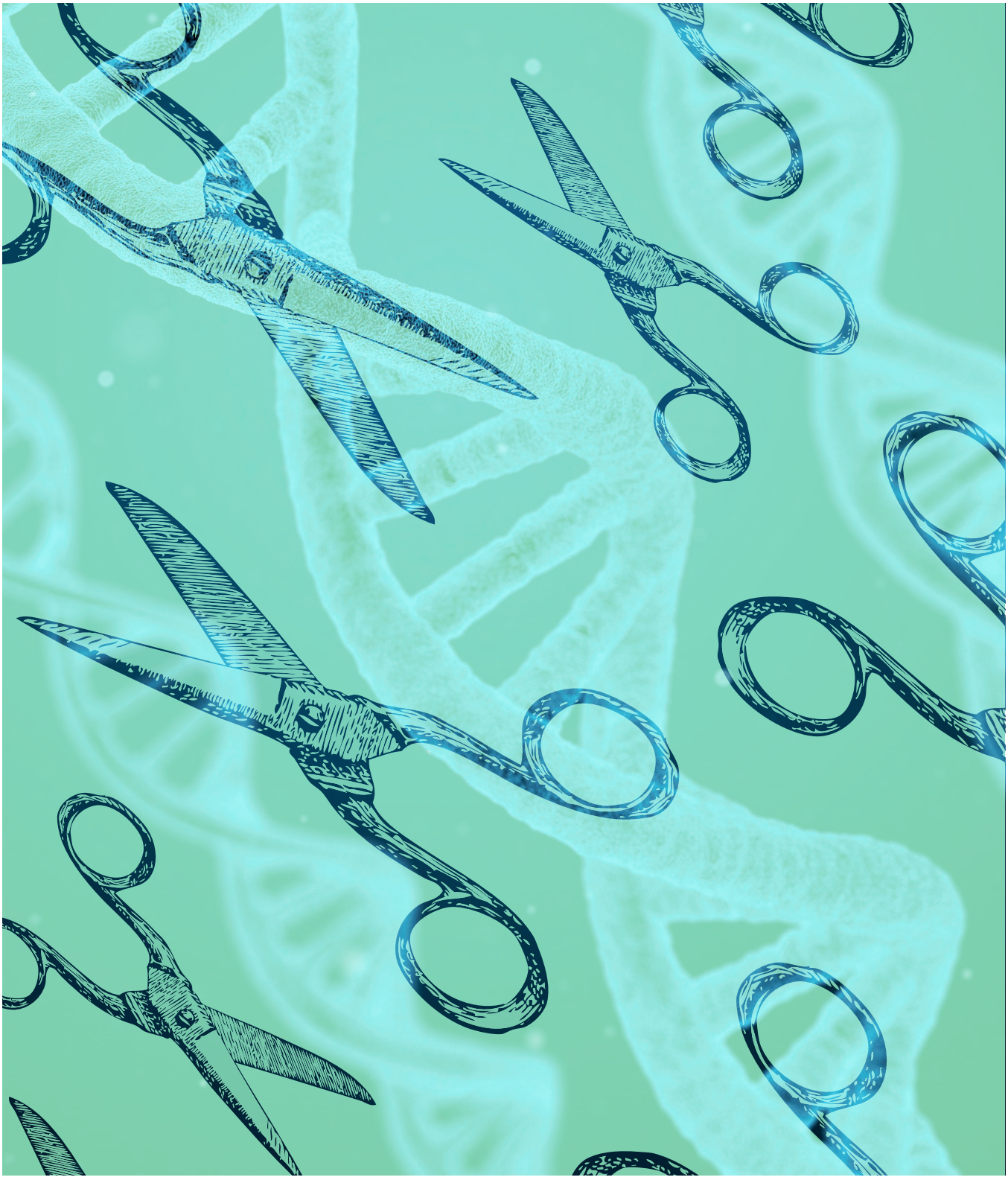
The quality trade-off

That's the plus side. The minus side is that it's not perfect; you have to worry about off-target effects when you're introducing chromosomal breaks. In our case – and many others – speed and efficiency really win out, and in that realm, CRISPR/Cas is superior to any other technique. But there's a dichotomy here between basic research and clinical applications. For basic research, we want the "quick and dirty" solution. We want to know the approximate answer as fast as possible, and then we'll keep investigating that until we can confirm it.

Clinical application is the other side of the coin. And there, all of a sudden, you really need to put on the

At a Glance

- *Genome engineering technology has existed for a long time, but our newest tool, CRISPR/Cas, is our fastest and most efficient yet*
- *The technique still needs refinement; some attempts work well and others not at all – but we don't understand why*
- *Solving those mysteries could give us a powerful tool for gene therapy and diagnostics*
- *CRISPR/Cas is so promising that I expect it go the way of PCR, becoming ubiquitous in all laboratories and – one day – clinics as well*



brakes. Even a single alteration that you don't want when you're putting cells back into a human patient could cause significant harm. So for that use, you need your gene editing to be as perfect as it can possibly be. We can afford some "sloppiness" in the system when we're performing basic research, but when it comes to patient care, that is – of course – unacceptable.

"Even a single alteration that you don't want [...] could cause significant harm."

The Lamborghini of gene editing

The easiest metaphor to describe other methods' relationship to CRISPR/Cas is that of a manual versus an electric typewriter, or a Toyota versus a Lamborghini. In principle, both of the former machines perform the same function: they allow you to type, or to drive from one place to another. But the latter are orders of magnitude better than the former. So CRISPR/Cas does the same thing as a TALEN or zinc finger (the best reagents that the field had previously utilized) – it just does it much, much better.

"Better," in this case, is mostly in terms of speed. To make a CRISPR/Cas reagent takes 24 hours; to make a good TALEN or zinc finger can take months of work. In that space of time, you can do dozens of CRISPR/Cas experiments! In fact, the difference is even more extreme because CRISPR/Cas allows you to easily multiplex. In the old days, we had to go after one allele of one gene at a time, an incredibly laborious approach when making double or triple knockouts because we had to do each round of gene targeting independently. With CRISPR/Cas, you can edit multiple loci at precisely the same time, cutting your workload by months – sometimes even years.

Your mileage may vary

One thing we have found out about CRISPR/Cas is that not every attempt works equally well. In some cases, we design a guide RNA (the portion of CRISPR/Cas that tells the complex where to make the break) that should go to a particular locus in the human genome, throw our reagents in with it, and it cuts beautifully; we get very high-efficiency repair and recombination. But if we make another guide RNA – even one that's fairly similar – we might find that it only kind of works. There's a little bit of repair and recombination, but not much, and we don't currently understand why that is. Annoyingly, we don't know why the process doesn't work with the same efficiency at all loci. I've had instances in my own laboratory where we've tried to engineer a locus and it just hasn't worked. We usually get around that dilemma by moving our guide RNA

Need to Know

What do potential CRISPR/Cas users need to think about before starting experiments?

1. Know how well your cell line takes up DNA.
   If you're working with a cell line that takes up DNA very well, you know that you're likely to be able to get your reagents into your cells – obviously a necessary first step in the process. If you're working with one of the more esoteric human cell lines that isn't very receptive to DNA uptake, that's an important thing to consider before you start.
2. Know how quickly your cell line proliferates.
   CRISPR/Cas editing works best in proliferating cells – but certain tissues are largely made up of non-proliferating cells. In those tissues, the editing process doesn't work very well. You need an S-phase DNA replication to push it along.
3. Know the ploidy of your cell.
   Ploidy doesn't matter as much with CRISPR/Cas as it does with other methods – but that doesn't mean you don't have to think about it. When we started gene editing, we always used diploid human cell lines, which are hard to get your hands on. Most human cell lines in culture have some sort of cancer background, and because of that, they tend to have more than 46 chromosomes each – which makes gene targeting very difficult. If you have four copies of the chromosome that carries your gene, you've got to modify all four of them. CRISPR/Cas is so much more efficient that aneuploidy isn't a dealbreaker anymore – but it is still relevant to the process of trying to figure out which cell line you want to use and which gene you want to alter.

up or down the chromosome just a little, and all of a sudden, we'll hit paydirt. I assume it has something to do with the chromatin state at the locus of interest – one region is more heterochromatic than another, or the DNA is so crowded with transcription factors that the CRISPR/Cas complex can't access it, or there are epigenetic modifications with which Cas isn't compatible. We just don't know; all we have right now is the empirical observation that CRISPR/Cas gene editing works better at some loci than at others. And additional technical issues – like cell lines that don't take up DNA very well – don't help matters.

So we have biological problems, and then we have technical problems layered on top of that. Between those two things, it seems almost impossible to predict how well CRISPR/Cas editing will work. You just have to do the experiment and see what happens. If it works really well, you jump up and down and then go on with your work; if it doesn't, you try to optimize. Is the problem the fact that the reagents aren't getting in? Is it that the enzymes aren't cutting very well? Can you use a better ratio, or combination of, reagents? There are plenty of ways to skin that particular cat – but it can sometimes take quite a while.

The bench-to-bedside barrier

When you start to consider using CRISPR/Cas for therapeutic purposes, it's somewhat of a different beast. We're no longer talking about culturing incredibly resilient cells that are easy to access and difficult to damage. If you want to re-engineer a patient's cirrhotic liver, there are all sorts of new issues. You've got to deliver the treatment to the liver without letting it reach other parts of the body; you've got to work in tissues made up of mature, non-proliferating cells; you've got to worry about efficacy and ensuring that enough cells are corrected to yield a therapeutic benefit. In some diseases, that might just require a 5–10 percent correction – but in others, you might have to modify most or all of the cells. And that's something we're not yet able to do.

"Once you start talking about therapy, you also need to start talking about ensuring the process is error-free."

Here, too, the issue of off-target effects rears its ugly head. In basic research, you can afford some sloppiness in your system; an extra double-stranded break here and there, for example, won't affect your experiment – but it can certainly affect the survival of a human being. So once you start talking therapy, you also need to start talking about ensuring that the process is error-free. But does that mean there's no hope for CRISPR/Cas in the clinic? I don't think so! In fact, it's already being used, and I expect that will only increase as we continue to develop the technique.

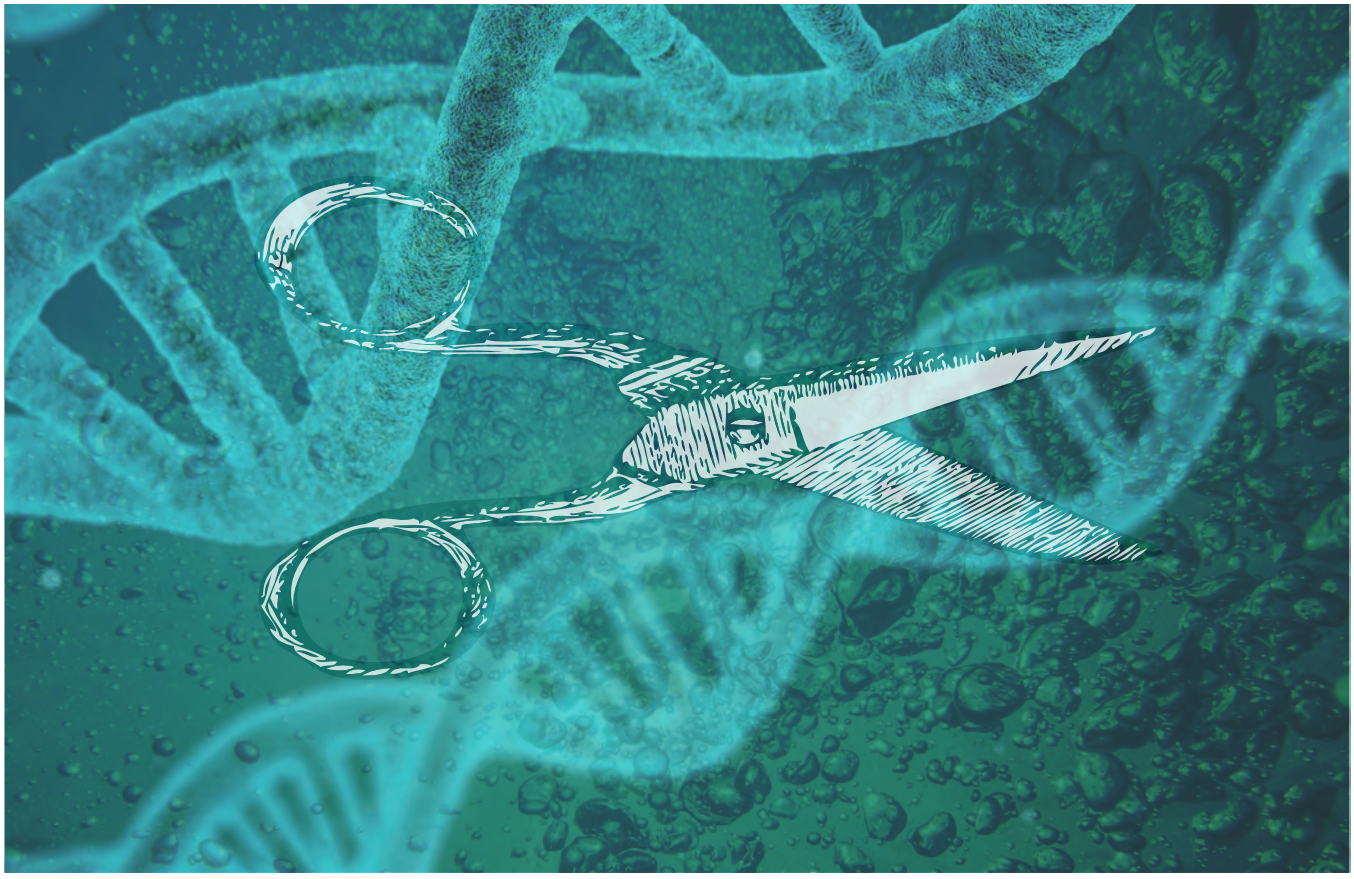
Reality check

Right now, I think there are two very clear applications. One is gene therapy. If you can get access to the tissues affected by a single-gene disorder, there's no reason you can't go in right now with CRISPR/Cas and change the "bad" DNA into "good" DNA. That's especially true of immunological or ophthalmic disorders, where the tissue is easily accessible. I think we're going to see enormous progress in the next 10 years or so as people start to attempt these kinds of gene editing treatments.

The other up-and-coming application is diagnostics. Human genome sequencing has essentially made it so that, within 20 years, every baby's DNA will be sequenced at birth. Doctors will examine each genome and say, "Oops, it looks like this child is at risk for diabetes," and offer preventative treatment. In order to do that, though, we're going to have to gain a much better understanding of the genome. At the moment, we have a lot of what we call "variants of unknown significance" – polymorphisms whose effects we don't yet understand. We can tell whether or not someone has a mutation at a particular locus, but we don't necessarily know whether or not that mutation actually has a biological consequence. Fortunately, CRISPR/Cas will allow us to answer those questions very quickly. Because it's so efficient, we'll be able to make 100 different cell lines where we've changed 100 different nucleotides, and then test all of them to see how their functions have changed. That's already ongoing, and in the very near future, it will allow us to figure out which pieces of DNA are biologically significant and which are not – and then, hopefully, fix those that are damaged.

Out of the "water bath" stage

It's always hard to imagine what the future might bring. If you'd asked me three years ago if we'd ever have reagents much better than TALENs, I would have probably said no – but all of a sudden, along



came CRISPR/Cas. Now, what's up-and-coming is the field of CRISPR interference and CRISPR activation. Essentially, you inactivate the Cas part of the CRISPR/Cas complex so that it no longer makes a break, but still localizes to a specific portion of the human genome. Then you can modify the Cas to have totally new functions, like methylating nucleotides or moving nucleosomes. Why is that useful? Well, for instance, you can produce phenotypic outcomes without actually changing the DNA – just by modifying the epigenetics to allow expression or inactivation. That's a very powerful tool, and it's one we're rapidly acquiring even now.

One of the scariest things about gene editing is just how fast the field is moving. The original Cas is already antiquated! It had a tendency to cut at low efficiency at extraneous sites, so laboratories have now developed more evolved versions with high-efficiency cleavage and reduced off-target effects – and that's only in the past few years. So there's no question that the system is here to stay. It's like the polymerase chain reaction (PCR). PCR arrived one day and simply never left; it's in constant use now. I see CRISPR/Cas the same way, and although there will be constant tweaks and improvements – just like PCR – I anticipate that, 20 or 30 years from now, everyone will still be talking about the technique and using it in their everyday work. It's hard for me to imagine anything so revolutionary that it would supplant CRISPR/Cas.

In terms of the PCR comparison, I think we've progressed just beyond holding our tubes in different water baths. In the beginning, it seemed like, every six months, somebody was inventing new and different PCR techniques. It just kept getting better and more efficient. That's where I think we are now with CRISPR/Cas – out of the water bath stage, but very much at the beginning of its evolution. We'll keep on finding new and different applications for it, and eventually, it will become as much of a laboratory standby as tools like PCR are now.

Eric A. Hendrickson is Professor of Biochemistry, Molecular Biology and Physics in the University of Minnesota's College of Biological Sciences, Minneapolis, USA.

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Seeing the Light

Conventional histopathology is destructive of biopsy tissue, and doesn't always provide enough information for accurate diagnosis and grading. "Slide-free histology" by light-sheet microscopy may change the game

Traditional tissue processing for histopathology calls for an array of protocols – fixing, cutting, staining – and cannot be done while leaving the sample intact. Not only does it require a lot of time and labor, it can also pose problems down the line if questions still remain – or if the pathologist's ability to make an accurate diagnosis is affected by the limited information available. Is there a better way? A group of researchers from the University of Washington think so – and they've developed a method of examining tissue samples nondestructively with light-sheet microscopy. Here, we speak with Jonathan Liu, Nicholas Reder and Lawrence True to find out more…

At a Glance

- *Standard analysis of biopsy tissue takes time and effort and can destroy samples, but the information it provides is not always worth the investment*
- *Digital and nondestructive microscopy approaches have been proposed in the past, but can actually increase the time and complexity involved*
- *Light-sheet microscopy works like a "tissue scanner" to efficiently image samples in 3D and add new diagnostic information*
- *The technique currently offers the same resolution as a 10X objective, but over extremely large areas without tissue damage and potentially with much less nucleic acid degradation*

What are the main issues with existing biopsy techniques?

Jonathan Liu: Standard histopathology of biopsy specimens is slow, labor-intensive, destroys the tissue, and can only generate a few limited 2D cross-sectional views. There are several nondestructive microscopy approaches, such as confocal or multiphoton microscopy, but they are typically slower and more complex for a clinician to use. Our custom light-sheet microscope is like a "flatbed scanner" for tissues; specimens can be simply placed on top of a glass plate and imaged from below. The technique is fast, simple and non-destructive because the tissue is not physically cut. In addition, our images are the same quality as standard histology – but in three dimensions. That has two major advantages: first, the entire biopsy can potentially be "sampled," rather than the tiny fraction possible with thin tissue sections on glass slides; and second, our volumetric imaging data can improve pathologists' ability to diagnose and stage lesions.

Nicholas Reder: Biopsies have enormous importance in healthcare. They often determine whether a patient receives a cancer diagnosis and, if so, what treatment is offered. But there are a few downsides to standard techniques, including the time and effort needed, the degradation of nucleic acids, and the result – 2D sections of 3D objects.

The one area where light-sheet microscopy stands alone is its ability to acquire 3D data. This ensures that there aren't any "gaps" in the image where the tissue is out of focus (a potential issue with other fluorescence microscopy techniques). And, especially exciting to me as a pathologist-in-training, the 3D information offers a unique view of the tissue – a whole new dimension for morphologists to explore and describe.

"We hope that our light-sheet microscope will give practicing pathologists a new tool."

Lawrence True: In some cases, traditional biopsy requires us to obtain and assess multiple sections to be certain of our diagnosis and the grade of the cancer. This takes time – up to several days – and consumes tissue to the extent that there might not be sufficient residual tissue for supplemental molecular studies. Our method avoids both of those problems.

How does your new light-sheet microscopy method for slide-free biopsy work?

JL: The majority of traditional microscopes use one common path for illumination and collection of light, which places constraints on imaging performance; for instance, the trade-off between field of view and depth of focus. With a light-sheet microscope, the illumination path and collection path are oriented at 90° to each other. The use of separate paths provides more flexibility to tune and optimize imaging specifications, such as resolution, field of view, and depth of focus. Another well-known advantage of light-sheet microscopy is that the illumination and collection of fluorescence light are extremely efficient, which improves sensitivity and reduces photodamage relative to other approaches.

NR: Light-sheet microscopy is a fluorescence microscopy technique that has gained popularity in the developmental biology and neuroscience fields. The tissue must first be labeled with fluorescent dyes before imaging. Then, the labeled tissue

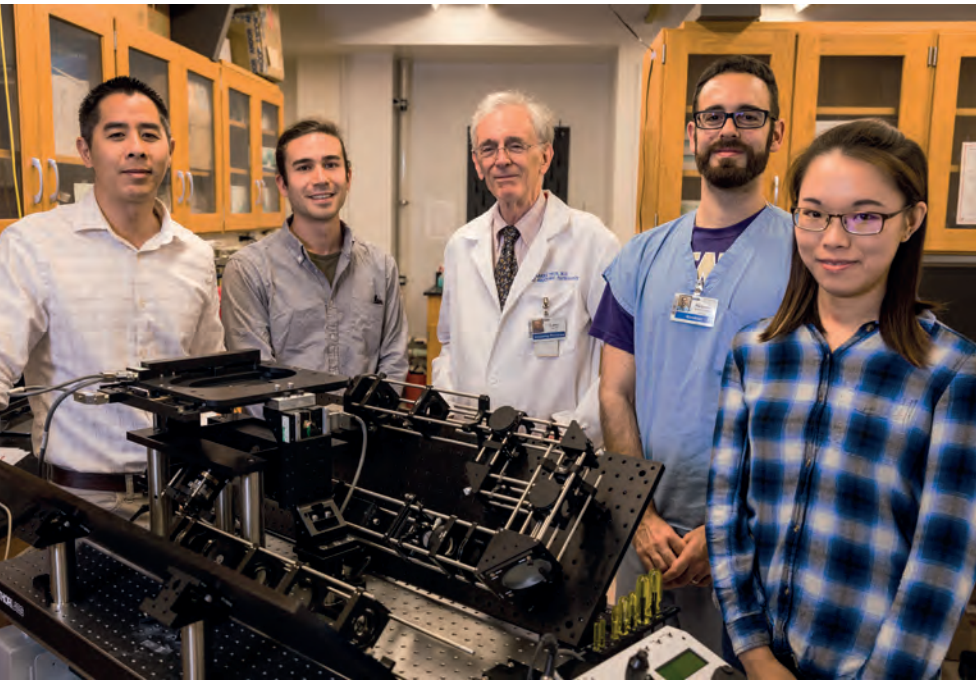
is placed on the microscope stage. The instrument uses a laser beam focused into a thin sheet of light that excites only the fluorophores within it, producing an "optical section." In contrast to cutting a thin, physical section of tissue, an optical section is nondestructive. A collection arm, arranged at a 90° angle to the light sheet, collects the emitted light onto the camera chip. The stage is scanned so that the entire surface of the specimen is imaged. Light-sheet microscopy's key feature is the ability to capture an in-focus image of a wide area with depth into the tissue. This enables 3D imaging when the tissue is rapidly scanned by the microscope. Other fluorescence microscopy techniques must scan the tissue point-by-point, which is far less efficient than light-sheet microscopy.

LT: We stain a fresh, not-yet-fixed biopsy core with two fluorophores, immerse it in refractive index matching solution, then image it on the light-sheet microscope. We can look at the digital images within 20 minutes of getting the biopsy, and we can even pseudocolor them to look like conventional hematoxylin-and-eosin-stained sections. Because no tissue is consumed or cut, it can later be used for supplemental studies like molecular mutation analysis – or fixed and stained as a routine specimen without compromising its quality.

How did you develop such a unique method?

JL: We were inspired by the great work of our predecessors in light-sheet microscope development. We simply adopted an already-successful technique for biological investigation and optimized and repurposed it in a custom design for clinical pathology applications. Why is it so popular? In a nutshell, few other microscopy techniques can image 3D volumes so quickly, or with such efficient use of light and fluorescence signal generation – vital for sensitive high-speed imaging with minimal photobleaching.

NR: The impetus to develop our light-sheet microscopy system was an unmet clinical need: the imaging of large areas of freshly cut tissue. My colleagues quickly realized that the system needed to image a large, irregular tissue surface, but also be fast enough to improve workflow. Light-sheet microscopy offered an attractive solution because it captures the entire tissue surface during a quick scan – something that isn't possible with techniques that have a small area of focus like confocal or multiphoton microscopy. Although light-sheet microscopy is quite popular, most commercially available systems are designed to image small, translucent model organisms without photodamage; they are not well-suited for clinical



specimens, which can be quite large and have irregular surfaces. Our system can accommodate specimens of many shapes and sizes, making it far more relevant to clinical practice.

3D imaging data from light-sheet microscopy has led to profound insights into developmental biology and neuroscience. We recognized its potential to improve diagnosis in clinical specimens – but fresh tissue is highly light-scattering and permits only a modest amount of depth imaging. To maximize the 3D imaging potential of light-sheet microscopy for clinical specimens, we developed clinically friendly techniques to clarify tissue. We expect that light-sheet microscopy of clinical specimens will add diagnostically useful 3D information, potentially leading to new insights in diagnostic pathology like those we have already seen in basic science.

How might the new technique change pathologists' day-to-day work?

JL: It should speed up and simplify the process of obtaining microscopy data from human tissue specimens. It will allow pathologists to interact with other clinicians in real time, for example to guide tumor surgeries or biopsy procedures. In addition, it will improve the accuracy of diagnostic determinations. We hope to build the technology so that pathologists need minimal training to prepare the specimens and operate the microscopes.

Going forward, we need advances in visualization software and computer-based analysis of our massive 3D datasets to allow pathologists to quickly make accurate tissue diagnoses. Fortunately, advances in 3D radiology (CT and PET), along with the explosion of research in machine learning and data science, will help to address these challenges.

NR: We hope that our light-sheet microscope will give practicing pathologists a new tool in cases where it's best to directly image fresh tissue rather than formalin-fixed, paraffin embedded sections. Biopsy adequacy, triaging for molecular testing, intraoperative consultations, and triaging in the gross room are all great initial applications for light-sheet microscopy.

One of the best aspects of our collaboration is that the two teams had such frequent and in-depth communication. Our engineering team's goal is to design devices that address unmet clinical needs, rather than to find homes for devices that have already been constructed. They made multiple trips to the pathology laboratory and sat at the multi-headed microscope to understand the needs of a practicing pathologist. On the pathology side, our main goal is to make the technology as familiar as possible to practicing pathologists. Thus, the biopsies are pseudocolored to look like hematoxylin and eosin. In addition, the microscope's open-top design makes it quite user-friendly, meaning that pathologists won't need in-depth training to use it. A major goal of our collaboration is to build a device that can be implemented in the clinic, which means minimal training and user-friendly equipment.

Can you describe an ideal situation for the use of light-sheet microscopy biopsy?

JL: We believe our technique is an improvement over conventional histology in all cases. It is particularly attractive in cases where speed is important (for example, to guide surgery in real time or to confirm the adequacy of a biopsy) or where 3D data adds value. To perfect the technique, we still need to improve its spatial resolution, depth, and imaging speed, and we need to optimize our methods of staining tissues in 3D to visualize molecular biomarkers of diagnostic importance.

NR: There are two "ideal" situations where light-sheet microscopy could make an immediate impact. The first is in patients with a known cancer diagnosis who are being considered for targeted therapy. Biopsies are obtained for genetic sequencing to determine which therapies might benefit the patient, but in-depth histologic analysis is unnecessary. In current clinical practice, we process biopsies using standard techniques that degrade nucleic acids. With our light-sheet

microscope, we can examine the tissue in the fresh state, preserving nucleic acids for high-quality sequencing.

The second is for rapid evaluation of surgical specimen margins during surgery. Currently, we either use frozen sections or, in some cases (like breast cancer lumpectomies), forgo microscopic evaluation altogether. Frozen sections have numerous downsides including tissue consumption, artifacts, and sampling errors. In contrast, light-sheet microscopy is nondestructive and can image the entire surface of large, irregular specimens.

I hope that our light-sheet microscope will give pathologists an additional tool for these scenarios – and that there will be many more "ideal" situations as the system continues to improve…

LT: With the new microscope, we can evaluate biopsies of tumors and margins quickly, thoroughly, and with less specimen artifact. It can be difficult to obtain tumor biopsies in some cases – for instance, when attempting to conduct mutation analysis; we don't always know when a sample has insufficient tumor material until the specimen is analyzed hours or even days later. Using a light-sheet microscope, we can assess the adequacy of the tissue quickly enough that, if necessary, we can request a repeat attempt to obtain sufficient tumor.

What are the next steps for this type of work – and what obstacles must still be overcome?

JL: We have a few steps to tackle:

- We will improve imaging performance to provide pathologists with images similar to those obtained using a 40X objective (typically the highest level of magnification used for conventional pathology);
- We will further optimize tissue clearing and staining protocols to improve their speed and the ability to image biomarker targets;
- We will work with computer and data scientists to improve the tools for visualizing and processing our microscopy data in a clinical setting; and
- We are starting to work on clinical studies to validate the benefits of our technologies for patients. At the same time, we're talking to other researchers and companies to improve and commercialize our methods so that they can make a difference in the clinic.

"If these technologies excite you as a pathologist, there are engineering groups who would be thrilled to learn from your expertise."

NR: The first obstacle to overcome is ensuring that the pathology workflow is enhanced rather than encumbered. This means working on our instrument's software and usability. We are collaborating with the University of Washington eScience Institute to build cloud-based solutions for data processing, management and visualization to address these needs. The next major step will be to construct a market-ready device and obtain FDA approval. Then, reimbursement is another major obstacle to overcome – but luckily, the College of American Pathologists (CAP) has a forward-thinking in vivo microscopy committee working with CAP's American Medical Association liaison to establish CPT codes for reimbursement. Once all of those pieces are in place, then pathologists will have a strong business case for purchasing the device – and positive experiences for early adopters will help widespread adoption in the future.

What advice do you have for pathologists wanting to adopt light-sheet microscopy?

JL: Talk to engineers in academia and industry. Try to partner with them to help these technologies become the standard of care one day.

NR: Engineers working in this field are eager to collaborate with pathologists. I would recommend surveying CAP's In Vivo Microscopy Resource Guide for attractive technologies – image interpretation is a good way to get started, because the images are digital and easily shared. Eventually, the goal of groups like ours is to have multi-site validation studies. In the near future, I anticipate opportunities for pathologists to have a device on-site and begin to acquire hands-on experience. The bottom line is, if these technologies excite you as a pathologist, there are engineering groups who would be thrilled to learn from your expertise.

LT: Consult optical and mechanical engineers. The College of American Pathologists is also planning to give a course, which could be a valuable resource.

Jonathan Liu is Associate Professor and Director of the Molecular Biophotonics Laboratory at the University of Washington.

Nicholas Reder is a Genitourinary Pathology Fellow at the University of Washington.

Lawrence True is Professor, Service Leader of GU Pathology, and Co-Leader of the Prostate Cancer Biospecimen Core at the University of Washington, Seattle, USA.

Babies' Breath

New research reveals a potential noninvasive predictor of bronchopulmonary dysplasia risk in preterm infants

By Jegen Kandasamy

Many prematurely born infants struggle with breathing, and as many as half may develop bronchopulmonary dysplasia (BPD), a lung function abnormality that stresses the infants' underdeveloped lungs and can result in lifelong chronic or even fatal disease. Reactive oxygen species (ROS) arising from prolonged oxygen therapy in babies who are unable to breathe sufficiently on their own interfere with the lungs' maturation and may mean that the terminal saccules – vital for gas exchange during breathing – don't develop correctly. But is there any way to predict which preterm infants may develop BPD, and therefore, which might need modified respiratory support? Until recently, the answer has been "no" – but a new type of biomarker may change that.

At a Glance

- *Preterm infants, especially those requiring prolonged oxygen therapy, are at risk of developing bronchopulmonary dysplasia*
- *None of the proposed genetic or cytokine biomarkers for BPD risk have been replicated upon closer study*
- *A new type of biomarker – mitochondrial function – may be more successful, and can be noninvasively tested in umbilical cord blood cells*
- *If validated, mitochondrial function testing could help doctors determine which infants need modified respiratory support*

Why we did it

Over the last few years, many medical professionals have hypothesized that pulmonary vascular dysfunction may be an important causative factor in the development of BPD in preterm infants. Even though evidence has now emerged regarding the central role of mitochondria in hyperoxia-related tissue injury – a key pathogenic factor for prematurity-related pulmonary disease – mitochondrial function is a relatively novel and under-investigated area in this disease process.

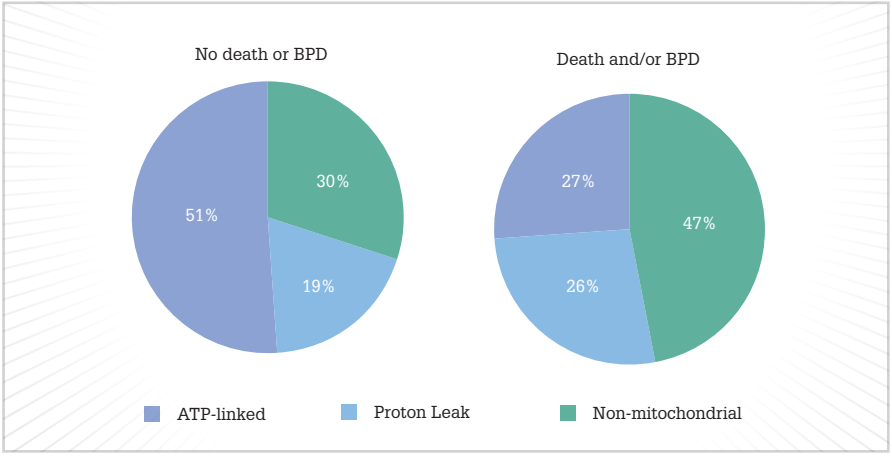
Human umbilical venous endothelial cells (HUVEC) have often been used as model systems to study the role of vascular function and pathology in the pathogenesis of several diseases, including diabetes, atherosclerosis and hypertension. But, until our study, they had never been used to investigate endothelial function as a risk factor for diseases to which the infants from whom they are obtained are susceptible in their earliest days.

We have collaborated in the past with our co-investigators at the University of Alabama's Department of Pathology on a project that used HUVEC obtained from term newborn infants to investigate mitochondrial bioenergetic differences arising from ethnicity. As a neonatal physician and a lung development and injury researcher, the approach intrigued me – and, as a result, I conceived the idea of using HUVEC obtained from preterm infants to measure endothelial function. My goal was to compare function between infants who later developed lung disease or died early versus those who survived without BPD. I was especially lucky to work at the University of Alabama at Birmingham, one of the few centers in the United States that has all the necessary components to work collaboratively on such a project – researchers with expertise in the areas of lung development and injury and in mitochondrial and redox biology research, as well as neonatal physicians and a robust and large neonatal intensive care unit. In the end, our study spanned four years, and I'm grateful to all of the people involved; without such a broad range of skills, we could never have completed our work.

Why could the results of the project be so revolutionary? At the moment, most scoring systems that predict BPD risk rely on variables like gestational age and birth weight differences, which contribute significantly to the developmental immaturity that places preterm infants at risk of complications. Those aren't always reliable measures, though, so researchers have made numerous attempts to identify potential biomarkers of these infants' risk of lung disease. Several studies have suggested various cytokines as possible biomarkers; others have proposed genetic polymorphisms (1,2). Unfortunately, no such link has been replicated in subsequent studies. In short, there has been no single reliable biomarker for predicting an individual's risk of developing lung disease. That's why our new discovery – that bioenergetic function (measured in cells that are relatively easy to obtain from preterm infants at the time of their birth) may be a marker for their risk of BPD – is so important. And, if successfully validated, it could improve our ability to identify prematurely born infants at increased risk before they develop significant lung injury.

How we did it

In our study, we harvested HUVEC from



the umbilical cords of 69 infants born at or earlier than 32 weeks' gestational age. We carried out bioenergetics measurements (intact HUVEC oxygen consumption) with a flux analyzer, as well as reactive oxygen species (ROS) measurements in hyperoxia-exposed HUVEC using fluorescence-based methods. Finally, we used quantitative PCR to measure damage to mitochondrial DNA.

Ultimately, we identified HUVEC bioenergetic function (measured as basal and maximal oxygen consumption under standard assay conditions) as the most significant factor that could reliably distinguish between infants with and without BPD. Fortunately, there are already several platforms that can reliably measure cellular oxygen consumption using as few as 1,000 cells – and we're currently in the process of developing a protocol that will use such systems to validate HUVEC bioenergetic measurements as a biomarker for BPD risk. The test won't hit the clinic tomorrow – validation will likely take at least two to three years – but, if successful, it would help us avoid the need for cell culture and allow us to measure endothelial mitochondrial bioenergetic function in primary cells obtained directly from the umbilical cords of infants at the time of their birth.

It's possible that, in the future, we might be able to test preterm infants for mitochondrial dysfunction in other ways. Mitochondrial genetic inheritance occurs through maternal transmission, so one particularly interesting approach would be test mitochondrial function and genetic differences using cells obtained from pregnant mothers, especially those at increased risk of preterm delivery. Sampling for this wouldn't be difficult; buccal epithelial cells obtained through oral mucosal scrapings would suffice. It's also possible to obtain human umbilical arterial endothelial cells (in addition to the HUVEC we used) from every newborn infant without the need for invasive procedures. These cells could also serve as a source of information regarding mitochondrial function and genetics.

Of course, along with the clinical test will come the question: who should be tested? In my opinion, any infant who is at risk for lung injury because of premature birth is likely to benefit from endothelial mitochondrial function testing. This is especially true for the various subgroups of infants that we particularly identified as having bioenergetic and redox dysfunction in our study – namely, those exposed to maternal and placental infection or inflammation and African-American infants.

What's next?

Our study identifies an association between BPD risk in preterm infants and the degree of their endothelial mitochondrial dysfunction. However, the mechanisms behind that association are still unclear and need further investigation. We have some preliminary hypotheses – for instance, endothelial mitochondrial dysfunction could cause deranged pulmonary angiogenesis by reducing nitric oxide and vascular endothelial growth factor availability. Then, the increased ROS generation from these cells could lead to the dysfunction of neighboring cells that constitute the pulmonary tree.

Because mitochondrial bioenergetic function depends on proteins in the electron transfer chain, which are derived from mitochondrial and nuclear gene expression, it's important to investigate both of these sets of genes. Variations in mitochondrial genetic haplotypes, differences in interactions between these two genomes ("mito-Mendelian genetics"), or both could impair or modify mitochondrial response to hyperoxia. Additionally, we also need to investigate mitochondrially targeted therapeutic strategies that could decrease pulmonary mitochondrial dysfunction – and thereby potentially also reduce the risk of lung injury in preterm infants. With a combination of better tests and better treatments, these babies may soon be able to breathe more easily.

Jegen Kandasamy is Assistant Professor at the University of Alabama at Birmingham and Director of the Rare Disease and Congenital Anomalies Programs at Children's Hospital of Alabama, Birmingham, USA.

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Time to Get Political

Grassroots politics can promote and protect pathology through advocacy and education. PathNET is getting pathologists involved and giving them a voice

Michael Schubert interviews Patrick Godbey

The last few years have seen some big political changes around the world, from Britain voting to leave the European Union to the recent US election. Political representatives come and go, but one thing is certain – doctors will always need to defend and advocate for the policies that best serve their profession and their patients. Here, Patrick Godbey describes the important work of PathNET, an organization dedicated to putting pathology's interests in front of US politicians to protect and promote our profession – and provide some guidance for anyone interested in getting more involved. Pathology needs you...

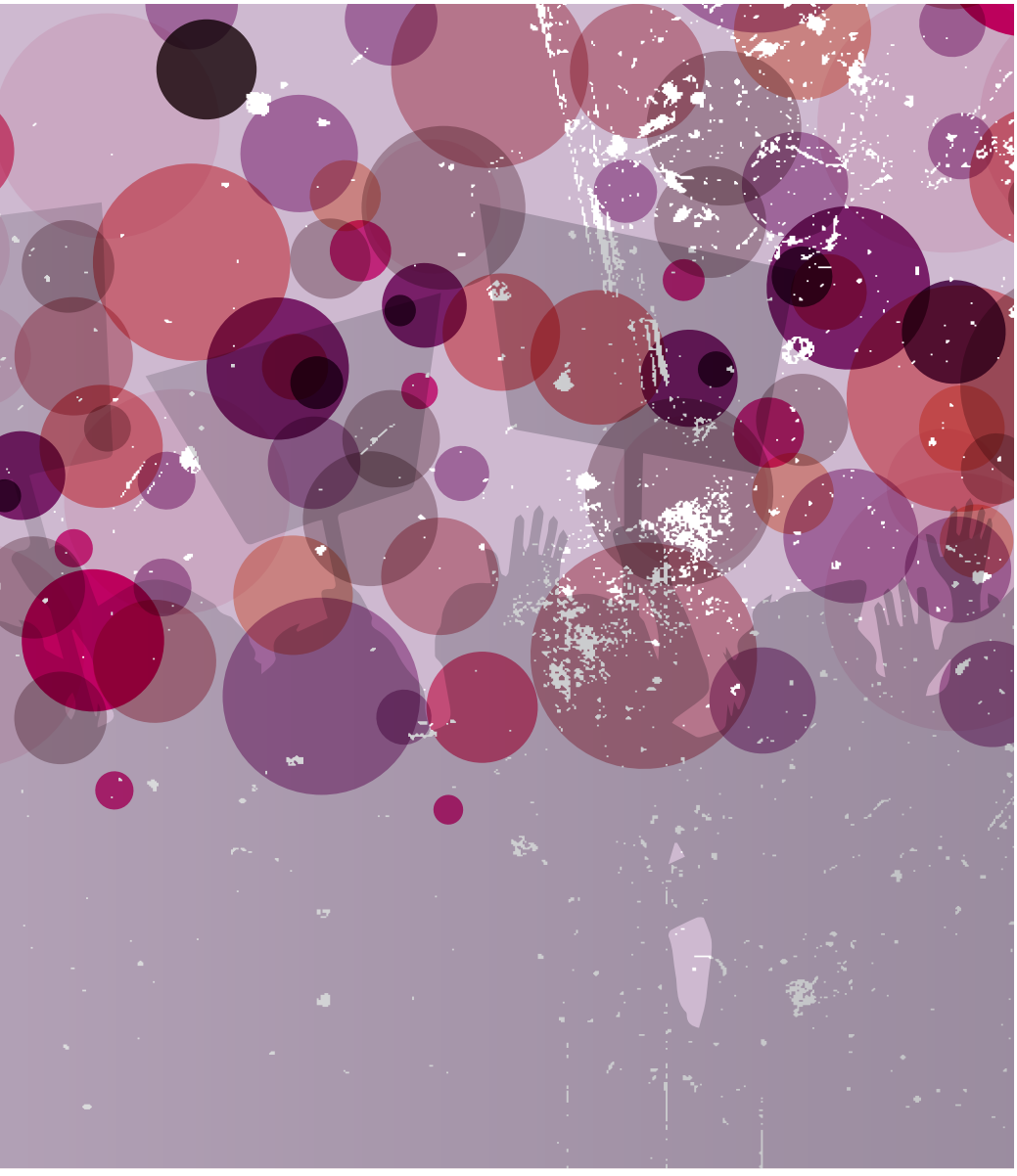
At a Glance

- *As a medical specialty with low visibility, pathology needs advocates – not just to inform the public, but to raise awareness in government too*
- *PathNET was founded to inform and educate pathologists, and to raise awareness of the issues affecting pathology among US politicians and policymakers*
- *The grassroots organization's many victories include helping to block controversial cuts and providing pathologists with exemptions from unhelpful policies*
- *From writing letters to inviting your local representative to tour your lab, there are many ways to get involved and make a difference*

Making pathology political

Recently, Godbey recounts, he and his wife were on a plane and a call was put out for any physician on the plane to come to the back. "I got up and left," he says, "and the lady sitting next to my wife asked, 'Is your husband a doctor?' 'Yes," she replied, 'he's a pathologist.' 'What's that?'"

It's a perfect example of pathology's visibility problem. Many pathologists have little contact with our patients, and the public is all too often unaware of laboratory medicine professionals' work. The College of American Pathologists



(CAP) is working to change this, and to show the world who pathologists are. But the focus shouldn't just be on the public – politicians and representatives need some education too.

The Pathology Advocacy Network – PathNET – is an organization that provides support and advice to help CAP members connect with their elected officials. It operates under the guiding principle that no one can advocate more effectively for the specialty of pathology than pathologists themselves, and its membership consists of around 2,300 CAP members. The resources it provides range from sending newsletters, to helping pathologists get in touch with their local congressman or senator, to helping them word letters to the editor. PathNET can arrange visits, meetings and conference calls, or let members know when their next town hall meeting or fundraiser is. Essentially, it helps pathologists get involved in politics in many ways, on both the federal and state level, and has a proven track record of successful advocacy.

CAP's headquarters are in Chicago, but there is also a very large and active CAP office in Washington, DC, which makes it easier to come into contact with elected representatives and administrative personnel. Just a few weeks ago, four CAP members were invited to speak with Tom Price, the Secretary of Health and Human Services. But there is also plenty of work to be done on a local level. Local representatives care about what's happening in their districts – they're interested in healthcare issues, and in patients. "My personal experience has been extremely positive," says Godbey, "and my local congressman has visited my lab four or five times."

"There are a great number of activities we can engage in to ensure our voices are heard".

Staff in the Washington office take care of the day-to-day running of the service, and the level of activity depends on what's currently happening – if there's a deal or a movement that PathNET is particularly interested in, they can ramp up their activity. Of course,

"Don't be afraid to get involved on every level."

some pathologists are more interested in politics than others, and some are quite proactive while others need more support. But CAP encourages people to take part to whatever extent they are comfortable with, and PathNET's members are all volunteers who are interested in making a political difference for our profession.

Pushing for transparency

Here's an example of how PathNET might respond to an important issue: first, it will receive information that a relevant bill or piece of legislation is being considered, and its members will identify relevant officials and follow them on social media. The next aim is to get as many people involved as possible, sending letters and emails and making calls. PathNET informs people of town hall meetings and encourages them to attend. Some officials have formal healthcare advisory committees they will go to for advice – members seek to serve on those. They'll visit Washington, DC and state capitals, and PathNET can help arrange the travel. CAP also has an annual policy meeting in Washington where PathNET members discuss their ongoing efforts. Godbey summarizes, "There are a great number of activities we can engage in to ensure our voices are heard."

A current issue for PathNET is Senate Bill 794, which is related to health insurance. The bill would require Medicare Administrative Contractors (MACs) and their carrier advisory committee meetings, where local coverage policies are discussed, to be open to the public and on the record. It would also require MACs to disclose the rationale and evidence being used to develop their local coverage determinations – and allow for appeal of these decisions, which is currently extremely difficult. This bill would greatly benefit patients, would lessen regulatory burdens on physicians, and would greatly improve transparency. Godbey and his fellow advocates are currently working to get more sponsors for the bill.

Getting signatures and fighting cuts

PathNET's previous successes? It stopped plans by the Centers for Medicare and Medicaid Services (CMS) to cap payment rates in Medicare's physician fee schedule, which would have resulted in significant pay cuts for pathology services. In turn, this

would have closed many labs, causing patients to lose access. The organization got involved and set up a campaign to block the proposal. Members made over 4,900 individual contacts with Congress, and sent thousands of comments to CMS. "I personally contacted one senator's office and explained that this proposal would result in greatly curtailed pathology services in south Georgia," says Godbey. "The senator's staff came to visit us, and a few days later I got a call saying that if I could provide some details for a letter, they would take it to senate and get as many signatures as possible – but they needed it quickly. I called CAP's people in Washington, explained the amazing opportunity, and asked for help. PathNET took up the challenge and 24 hours later the letter was in the senator's office. Capitol Hill responded, and the letter got 42 senator's signatures, from both Republicans and Democrats. In short, we played a significant role in stopping a cut that we believed was unjustified."

Another example Godbey gives is the introduction of Medicare payment penalties for physicians not using electronic medical records (EHRs). "Pathologists and labs don't use EHRs; we use laboratory information systems, so it was impossible for pathologists to comply with the new rule, which would have resulted in significant penalties. But again, we conducted a campaign that got a great result – over 100 members of Congress sent letters to CMS calling for pathologists to be relieved of this requirement. And so in large part because of PathNET's efforts, pathologists are exempt. Both cases show that grassroots campaigns really can have a big impact."

Anyone can make a difference

With all the changes going on in the USA today, Godbey sees PathNET ramping up its activity and continuing its good work going forward. "For any pathologists interested in politics, both in the US and elsewhere, my advice would be this: get involved! Attend meetings and fundraisers, and make yourself known. Tell your politicians, 'I am a pathologist from your district, this is my name, and here are the issues that concern me.' Develop a relationship, not just with your local politician but with your representatives on a higher level – in the case of the US, know your federal officers, your state representatives, and your senators. Don't be afraid to get involved on every level."

It may sometimes feel like an uphill struggle, he says, but individual pathologists truly can make a difference. If you're thinking, "I practice in a small town in a rural area, and I can't do it" – Godbey responds: "You can! Get involved and advocate for yourself, your profession, and your patients."

Patrick Godbey is a practicing anatomic and clinical pathologist with Southeastern Pathology Associates and Southeast Georgia Health Systems, Georgia, USA. He is a governor of the College of American Pathologists, and Chairman of the Council on Government and Professional Affairs.

Kallikrein Dream

Sitting Down With...
Eleftherios P. Diamandis,
Hold 'em for Life
Chair in Prostate Cancer
Biomarkers, Head of Clinical
Biochemistry, Mount Sinai
Hospital and University Health
Network; Professor & Head,
Division of Clinical Biochemistry,
Department of Laboratory Medicine
& Pathobiology, University
of Toronto.

How did you first become interested in cancer biomarkers?

My involvement goes back 35 years. In the 1980s, there was a lot of interest in new biomarkers, and there was a flurry of activity with the discovery of prostate specific antigen (PSA), and a number of other markers, like CA 125 for ovarian cancer, CA 15-3 for breast cancer, and CA 19-9, which is used for pancreatic and colon cancer. As a young biochemist, I was fascinated by all these new discoveries, and naturally wanted to get involved.

When PSA was discovered, the general consensus was that the family of PSA genes included three members. But in the early 1990s, there were new reports describing genes homologous to PSA in the same genomic region. So we developed a hypothesis that there may be other undiscovered members of this family. We initiated a genomic effort to find them, and were surprised to find a whole family, not of three genes, but of 15 different genes on exactly the same genomic locus on chromosome 19. We subsequently cloned, characterized, and named the enzymes that they code – serine proteases that belong to the kallikrein family. And I'm still studying them to this day.

What makes the kallikreins so fascinating?

Many researchers are looking into their biological function. They appear to be very nice biomarkers not only for prostate cancer, but also ovarian, lung and other cancers. And we're only now beginning to understand what these genes – and the proteins they produce – are doing. For example, we're now convinced that the kallikreins participate in diverse biological functions, such as semen liquefaction, in which the major player is PSA. They also play a major role in the cascade of events involved in skin desquamation and regeneration, and we've found them in cervical fluid and in sweat. Kallikrein 6 is highly expressed in the brain, and we suspect it may play a role in the development of neurodegenerative diseases such as Alzheimer's. It's becoming clearer and clearer that these proteins have diverse functions in various parts of the body.

What do you look for in a potential cancer biomarker?

Most of the markers we currently have in the clinic are used for monitoring previously identified cancer patients, to see if their therapy is working. Unfortunately, this means the impact of existing markers is relatively small. We need to look to population screening and find something that we can test for in asymptomatic individuals. The impact this could have on clinical care is huge. If we can detect cancer early and implement effective therapies much earlier, this could make a big difference to patient outcomes.

Which of your current projects are the most exciting?

We are working to develop assays to measure a small number of tissue-specific proteins. It's an area that hasn't really been looked into before, and we're hoping to identify their clinical value, as we suspect that they have hidden potential.

We've also just published a paper in which we put forward the idea of creating a database of personalized cancer biomarkers that are useful in different patients. We have named them rare markers – markers that may be highly useful, but only in a few patients. We think this could be another exciting new avenue. For the last 30 years we have tried to find one biomarker that will work for all patients. But molecular studies show that different types of cancer are not specific diseases – breast or ovarian cancer is actually a group of related diseases with different molecular features and signatures. And that means we have to accept that finding one biomarker to work for all of these patients is not very realistic. We believe that the way forward is identifying rare biomarkers and developing repositories for people to report them – eventually, we could create a rich enough database to look up a biomarker for any patient.

Do you have any advice for newcomers to the field?

I'm actually in the middle of preparing a new lecture on mentorship, and I do think it's important to share your experiences with younger people. An important tip is to be honest with your science. There is a lot of press lately on fabrication of results – this is totally unacceptable. It doesn't build careers; it destroys them. So I always tell my students: never consider fabrication. Don't go there. Be honest with yourself and with your work.

I would also say: work hard, develop multidisciplinary approaches, and read widely to expand your knowledge. But don't forget to have interests and passions outside of science. I don't want to create robots with tremendous output, but to develop human beings who enjoy life. Finally, be persistent – don't be discouraged by failures. If you get 99 failures and one success from 100 attempts, embrace it! Learn and move forward.

If you weren't a scientist, what would you be?

I've had a great deal of fun exploring new knowledge – and it's a privilege to work with very talented young people. My number one alternative would be a musician; however, though I love listening to music, I have no talent for making my own... Without science, I'd probably have chosen something athletic – perhaps a tennis player. But given how wonderful and rewarding my career has been so far, I don't think I'd change it!

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