Tissue issues

Millions of tissue samples have been collected and archived, but researchers wanting to explore them at the molecular level have found it tough going. Nathan Blow investigates the issues.

According to experts, there are more than a billion tissue samples archived in hospitals and tissue banks around the world, most of them formalin-fixed and paraffin-embedded (FFPE). Today, these samples present both an incredible opportunity and a huge challenge to researchers. FFPE tissue samples have been extensively annotated and well preserved, allowing detailed study of the progression of diseases such as cancer. But due to the method of preservation, obtaining biomolecules from these samples is proving difficult, to say the least.

FFPE was first described more than 100 years ago, and most hospitals still use this method today. But there has never been a set of standardized guidelines for processing FFPE tissue samples taken from patients to preserve tissue histology, let alone biomolecules. And although it works well for histology, the lack of standardized guidelines seems to have hampered the use of FFPE samples in molecular analyses. This may soon change, as pathologists are working towards standardizing FFPE sample preparation, and companies and researchers are developing the technology needed to isolate biomolecules and tap into the vast treasure chest of archived samples.

Although FFPE tissue preparation is simple in theory, many problems associated with downstream molecular applications — such as PCR or microarray analysis — can arise. "This is all about the fact that there has been no attention paid to uniformity of preparation," says David Rimm, a pathologist at Yale University. Between hospitals the time to tissue fixation and even the method of fixation can vary dramatically.

D. RIMM

David Rimm: time is key in tissue analysis.

"I would say that the biggest issue is time from ligation of circulation to fixation," says Rimm. During this period of ischaemia, molecular changes occur that cause problems in obtaining biomolecules. "Phosphorylation is very sensitive to ischaemic times. There seems to be promiscuous phosphotases in the cell that knock phosphates off tyrosines during this period," says Rimm. DNA and RNA can also suffer damage before fixation, with enzymes degrading and modifying both.

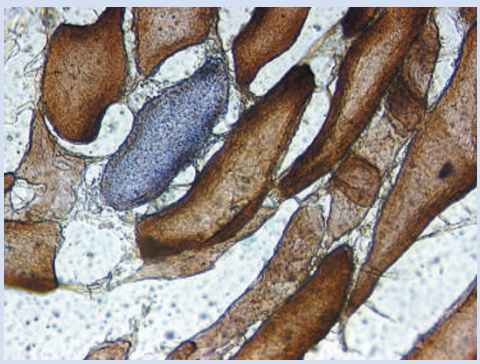
It seems obvious that rapid fixation is the answer, but no simple solution is in sight. Rimm says that for a researcher interested in only DNA or RNA, rapid fixation using quick freezing methods, instead of chemical fixation, is probably best. But if you are interested in proteins, freezing is problematic as the subsequent thawing process tends to break up and denature proteins and, like ischaemia, can lead to modifications.

Then there are pathologists who think that standardizing the time to fixation will prove to be a difficult task. "Standardization would be great, but I don't know how realistic it is across institutions or even within institutions," says Christine Iacobuzio-Donahue, a pathologist at Johns Hopkins University School of Medicine in Baltimore, Maryland. She believes that the best place to tackle the problems caused by ischaemia is actually downstream of fixation,

FROZEN IN TIME

Analysing proteins from tissue samples that have been formalin-fixed and paraffin-embedded (FFPE) can provide critical information about how cells function before fixation. "When you formalin-fix the protein, you fix it in time; it is not going anywhere," says Peter Tunon, vice-president of sales and marketing at Expression Pathology in Gaithersburg, Maryland. Researchers are taking advantage of this fact to explore the protein world in FFPE samples.

Expression Pathology was founded in 2001 by researchers from the US National Cancer Institute and the company Life Technologies (now Invitrogen) who had experience in studying gene expression in tissue and histology. "The company was founded on the fact that examining protein expression is crucial to understanding what is happening in cells," says Tunon. To this end, Expression Pathology has worked on ways of extracting and isolating proteins from FFPE tissue samples for analysis by mass spectrometry or reverse-phase dot blot arrays. The company developed a technology that integrates extraction of total proteins from FFPE samples with tryptic digestion so that finished samples are ready for mass spectrometry. To simplify the system further, all reagents are completely compatible with mass-spectrometry instrumentation, says Tunon, making it a good starting point for broad-based screening of proteins in FFPE samples. Other companies also think that protein isolation from FFPE tissue samples will provide valuable information. QIAGEN and EMD Chemicals, both based in San Diego, California, now offer systems that chemically reverse formalin crosslinking to isolate full-length proteins for applications such as western blotting and protein arrays.



LEICA MICROSYSTEMS

Caught on camera: a section of human muscle after dual histochemistry.

Surprisingly, post-translational modifications (PTMs) such as phosphorylation and acetylation, can also be observed when examining proteins isolated from FFPE samples. PTMs can be critical to the role of a protein in the cell, changing the function or localization. "We do see post-translational modifications that are preserved on the peptides, and they seem to be present in ratios that are similar to those we get when using fresh frozen tissues," Tunon says of work done by Expression Pathology.

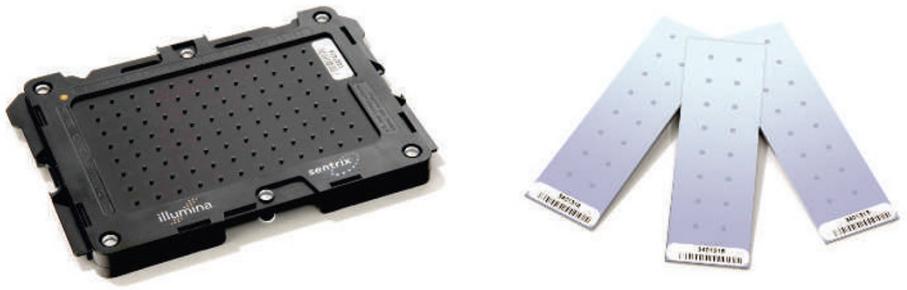
Even much older archival samples of FFPE tissues do not seem to pose a problem for protein extraction. "We have worked with samples that are more than 15 years old and found no difference in the profiles compared to samples that were just a few weeks old," says Tunon. He is quick to add that he believes even older samples could be examined, but Expression Pathology has yet to test this idea. N.B.

after the biomolecules have been isolated. "Once you have the samples and have extracted the biomolecules you are interested in, then you can perform quality control to determine if all your samples are similar," she says.

Even if the optimal time to fixation were found and standardized, this is only part of the problem; researchers still need to identify the best fixative to preserve both histology and biomolecules. Formalin, the most widely used fixative, provides good preservation of tissue histology but can cause problems for researchers interested in downstream molecular applications. "Formalin tends to crosslink the tissue to such an extent that it is hard to get fragments of DNA that are longer than, maybe, a hundred or two hundred base pairs," says Rimm. Formalin fixation causes the crosslinking of DNA as well as RNA and proteins, although proteins seem to fair better following formalin treatment (see 'Frozen in time').

Preservation society

Although tissue preservation has its problems, the good news is that these are now being dealt with. Governmental agencies, such as the US Office of Biorepositories and Biospecimen Research at the National Cancer Institute (NCI) in Bethesda, Maryland, are starting to tackle the difficult issue of biospecimen standardization. In June, the office released a guide to the NCI's best practices for biospecimen resources, detailing technical guidelines for NCI-supported biospecimen collection and storage. And researchers and companies are now creating methods to use the degraded and modified biomolecules obtained from FFPE samples for molecular analysis.



ILLUMINA

The DASL assay can be processed on two different platforms: the Array Matrix (left) or the BeadChip.

Isolation of RNA or DNA from FFPE tissue samples can be accomplished using a number of methods or kits. The main problem is that, almost without fail, the RNA or DNA isolated is degraded and chemically modified. But because researchers want to tap into the vast archives of FFPE tissues for global expression analysis and biomarker discovery, this is spurring companies to address the issue.

Microarray analysis has proven a valuable tool for understanding global gene-expression patterns. But using the degraded messenger RNA obtained from FFPE tissues for such analysis is problematic. "The results on standard microarrays are currently unsatisfactory," says Shawn Baker, scientific product manager for gene expression at Illumina in San Diego, California. For this reason, Illumina offers a gene-expression application that can study RNA extracted from FFPE tissue samples. Called DASL, short for cDNA-mediated annealing, selection, extension and ligation assay, this system amplifies the mRNAs from FFPE-extracted samples, but unlike other amplification systems it is not 3′ biased, says Baker. "We use a combination of random and oligo dT primers to generate the complementary DNA, which means that even with degraded RNA it still amplifies quite well and produces good, consistent profiles." Following the first amplification, the DASL system uses two gene-specific probes to amplify the cDNA. The resulting cDNA can be hybridized to a DASL-specific array. "The DASL assay is multiplexed up to 1,536 genes. So you get a tremendous boost in the overall throughput," says Baker.

Although unable to survey as many genes as standard microarrays, Baker says that using the DASL system, researchers have been able to profile FFPE tissue samples that are up to 30 years old — demonstrating the potential to

THE CUTTING EDGE

MOLECULAR DEVICES

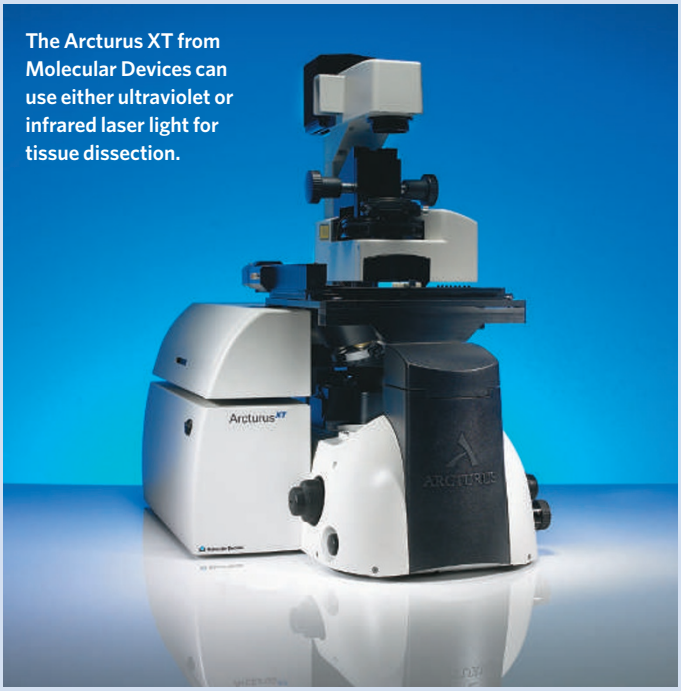
Researchers have the opportunity to play surgeon — slicing and dissecting out specific sections of tissue or even cell populations — with laser-capture microdissection (LCM). This level of analysis might seem to be difficult when applied to formalin-fixed and paraffin-embedded (FFPE) tissue samples, but many companies are now offering easy and quick LCM solutions.

Leica Microsystems of Wetzlar, Germany, offers the LMD6000 LCM system, which uses an upright microscope for dissection and capture. Christoph Horlemann, the company's product manager for the LMD6000, says this is possible because the transport mechanism for capture is based on gravity, unlike other LCM systems on the market.

'Transport mechanism' refers to the method for delivering a dissected tissue sample from a slide to a collection vessel. Other approaches to this process include that of PALM Microlaser Technologies in Bernried, Germany, which uses a 'pressure catapult' to send sections into tubes from the LCM instrument, and the CapSure system from Molecular Devices in Sunnyvale, California, which uses the laser to extend a polymer onto the tissue sample for capture.

Leica Microsystems' transport mechanism uses slides with a foil covering. The tissue is attached to the foil covering but not the slide, so researchers cut the tissue and the foil together and the sample simply falls by gravity into the collection tube below, explains Horlemann. But even this foil coating might not be needed in the future.

Both Leica and PALM are working with Expression Pathology of Gaithersburg, Maryland, on what may be the next generation of LCM slides. Called Director, these glass slides are based on laser-induced forward transfer (LIFT),



The Arcturus XT from Molecular Devices can use either ultraviolet or infrared laser light for tissue dissection.

LEICA MICROSYSTEMS

examine the numerous collections of archived FFPE samples. Illumina is now working to increase the number of genes analysed for each DASL assay.

NuGEN Technologies in San Carlos, California, is another company developing methods to use RNA extracted from FFPE tissue samples for gene-expression analysis. NuGEN specializes in working with very small amounts of RNA or difficult-to-use RNA, such as RNA extracted from whole blood or very degraded sources. This is commonly seen with clinical samples, but is most significant in the case of FFPE, says Gianfranco de Feo, senior director of customer solutions at NuGEN. Although NuGEN did not start off looking at RNA from FFPE tissue samples, it was the next logical step. "We have had a product on the market for over a year now that allows users to work with very degraded RNA in very limited amounts, down to 500 picograms. We built on that technology to create kits for the much more degraded RNA that comes from FFPE samples," says de Feo.

At the core of NuGEN's technology is its amplification and labelling system, which has been optimized to work with Affymetrix 3′ microarrays. The system relies on a combination of random hexamers, similar to that of Illumina, augmented with oligo (dT) primers to convert mRNA into cDNA in a linear amplification process. The inclusion of oligo (dT) primers was essential because the Affymetrix arrays probe the 3′ ends of transcripts. But NuGEN hopes to have labelling and hybridization protocols and products for other microarray platforms available before the end of the year.

To determine how well these degraded RNA samples from FFPE tissue will work on microarrays, NuGEN developed a tool using quantitative real-time PCR (qPCR) assay. It turns out that the results from this assay correlate very well with the overall results of microarray analysis, says de Feo, allowing researchers to decide whether the data that could be obtained from the array will be of sufficient quality to continue. And this is critical information, as in some cases less than 50% of the transcripts on the array may hybridize with the amplified RNA.

Think small

Asuragen in Austin, Texas, is a new company on the commercial block, working to understand and characterize the biological role of small RNAs. Although founded only a year ago, Asuragen's RNA roots go much deeper. Asuragen is a spin-off of Ambion, a company that worked in the field of molecular biology with a focus on RNA for nearly 17 years. "At Ambion, we developed the first kits and technologies for characterizing small RNAs," says Gary Latham, associate director of technology development at Asuragen. "And when microRNAs emerged as a new class of regulatory RNAs in humans, we were sitting in an excellent position to explore this area of 'biological dark matter'." In March 2006, Ambion was sold to Applied Biosystems for US$273 million and a portion of those proceeds were used to fund Asuragen.

Asuragen has concentrated its efforts on the diagnostic and therapeutic opportunities of microRNAs (miRNAs). "As they are smaller, miRNAs tend to survive the more tortuous conditions of FFPE tissue processing better than mRNAs," says Latham, which makes

Leica Microsystems offers the LMD6000 for laser capture microdissection applications.

a non-contact microdissection technique that uses a thin energy-transfer coating that replaces plastic films or adhesives. The technology was co-developed by researchers at the US Naval Research Laboratory in Washington DC and scientists from Expression Pathology.

Laser energy is transferred to the transfer layer and the layer is vaporized. The laser energy is then converted into kinetic energy, and the selected feature is shot instantly into a collection tube.

LIFT works equally well whether the tissue is collected up (PALM) or down (Leica) as the energy is sufficient to propel the tissue into the collection tube either way. As the transfer layer completely absorbs the laser energy, the biomolecules in the sample are not affected. The use of glass with this coating has other advantages as well.

"Glass slides are quite useful as you can also perform fluorescence and contrast applications without any interfering foil," says Horlemann.

Molecular Devices, a division of MDS Analytical Technologies, acquired Arcturus in 2006 and is now providing both the Veritas and the Arcturus XT LCM systems. Whereas most LCM systems use an ultraviolet laser for cutting samples, the systems offered by Molecular Devices can have either ultraviolet or the standard infrared laser options.

"The infrared laser is ideal for small areas where a user is looking to pick up only a cell or two," says Steven Blakely, product manager for the Arcturus LCM systems. "Infrared allows for a gentle collection of cells."

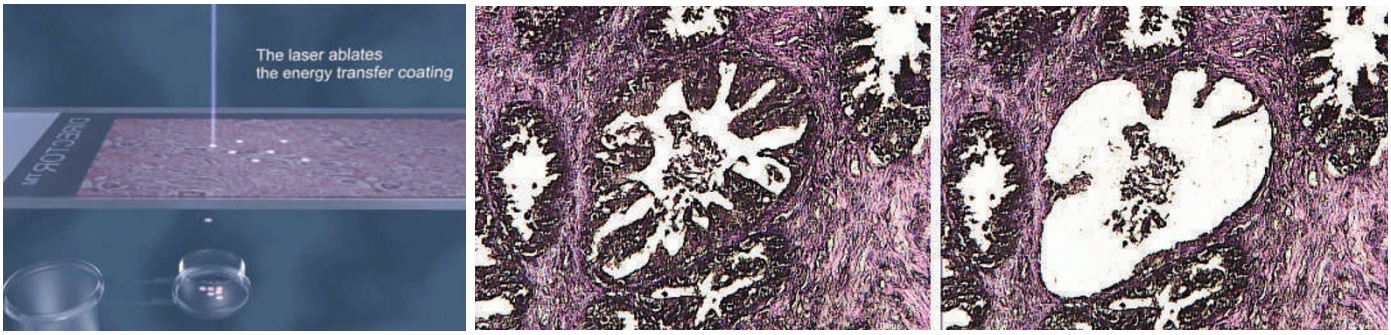
The use of infrared laser light is critical to the CapSure system because the dye found in the CapSure polymer is activated and becomes adherent with infrared light. Blakely says the CapSure system is particularly useful for FFPE samples that are already mounted on glass slides because the transport mechanism involves only the polymer adhering to the tissue and so can work with any glass slide.

Cutting tissue samples with ultraviolet light offers advantages too, such as dissecting thicker samples. Leica has worked to optimize objectives for its LCM systems with high-energy ultraviolet-light transmission, allowing more power to come in direct contact with the tissue sample. Horlemann says this provides faster cutting of the thicker tissue samples while using less power.

The future of LCM might just lie in automation and increases in throughput. "Now, researchers want to do faster, automated microdissection for proteomics," says Horlemann. But before anything else, some hardware and software issues needed to be resolved.

Any automated software package has to control all microdissection steps including focusing the microscope, recognizing cells of interest, focusing the laser and defining the area to cut — definitely not a simple task, but one that companies have worked on and made tremendous strides in recent years. Horlemann says that new advances are making automation easier every day. He points to the Director slides, which can allow for contrasting methods and fluorescence, making it easier when attempting to automatically define cells of interest as a step in the right direction.

Although simpler now, further advances will be required before LCM becomes as easy as pushing a button and walking away. N.B.



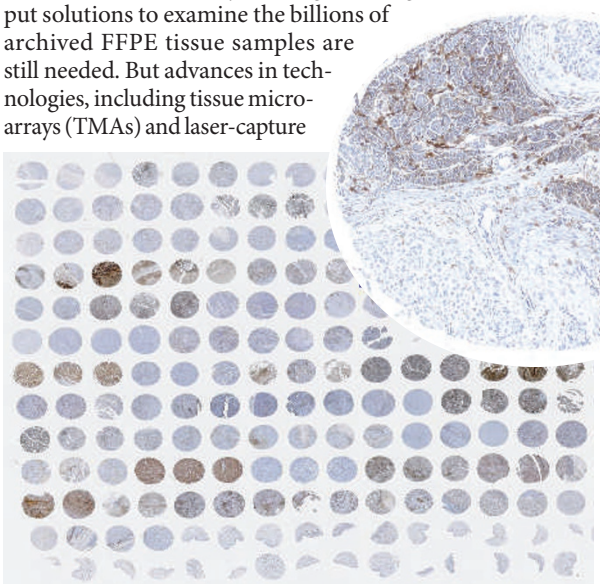
EXPRESSION PATHOLOGY

Direct action: Expression Pathology's Director slide (left) in action, and tissue samples before (middle) and after laser capture microdissection with the system.

degradation less of an issue. This means that downstream applications, such as microarrays, are better suited to miRNAs isolated from FFPE samples than mRNAs, says Latham. "We have found that there is tremendous diagnostic potential for microRNAs as biomarkers of disease states," he adds. This shows how companies are moving from traditional RNA analysis to non-traditional methods that use FFPE tissue samples.

Several other companies also offer technologies to explore RNA isolated from FFPE tissue samples. Panomics in Fremont, California, has developed a direct hybridization method using branched DNA technology. The system, called QuantiGene FFPE, is unique because it does not involve the linear amplification of RNA, as do many other systems. Instead, the use of branched DNA technology permits the direct measurement of RNA from the sample source. The Paradise System, developed by Arcturus Biosciences and now supplied by Molecular Devices of Sunnyvale, California, has been optimized for RNA extraction from FFPE samples, followed by a linear amplification step prior to use in qPCR or microarray applications.

Even though much headway has been made in the molecular analysis of FFPE tissue samples over the past few years, high-throughput solutions to examine the billions of archived FFPE tissue samples are still needed. But advances in technologies, including tissue microarrays (TMAs) and laser-capture microdissection (see 'The cutting edge'), are signalling that high-throughput analysis might be around the corner.



BACUS LABS

Tissue microarrays containing single spots of tissue (inset) provide a high-throughput histology solution.

In 1998, Juha Kononen and his colleagues described a tissue sampling method that produced regular-sized spots that could be densely packed on a microarray slide (J. Kononen *et al. Nature Med.* **4,** 844–847; 1998). Using this methodology, archival FFPE tissues can be sampled onto TMAs, allowing researchers to examine numerous samples by techniques such as fluorescence *in situ* hybridization and immunohistochemistry on a single microscope slide. Nucleic acids and proteins can even be extracted from archival FFPE tissue TMAs. Best of all, the technology for constructing TMAs is readily available from commercial suppliers.

Spot the tissue

Beecher Instruments of Sun Prairie, Wisconsin, produces both manual and automated tissue microarrayers. With the Manual Tissue Arrayer II, a block can be directly attached to a microtome for sectioning of arrays, whereas the automated arrayer, ATA-27, accepts nearly all tissue cassettes and can be adapted for either large arrays or variously shaped archival samples. The ATA-27 can also accommodate a full range of tissue spot sizes from 0.6 to 3 millimetres.

One concern regarding TMAs has been the representation of a whole tissue section by a single spot. "As the throughput is so much higher, I think that trumps the fact that it is not a whole section," says Rimm. But he cautions that care must be taken when constructing tissue microarrays. The optimal region to be sampled for the microarray is usually identified by a pathologist, but only a section or core of this region can be placed into the array. Problems can arise because the region that cores are taken from can vary. A core taken from the leading edge of a tumour might provide different results from a core acquired from the middle. So design of the slide is crucial; spots should be separated and standards (spots of known tissues) used.

In the past, pathologists have evaluated and analysed TMAs following immunohistochemistry or other histological analyses. But that is now changing, and some companies are offering software specifically for automated TMA analysis.

Image bank

Bacus Laboratories in Lombard, Illinois, and recently acquired by Olympus, is one such company. It has focused its efforts on virtual microscopy — the digital imaging of microscope slides — which involves scanning an entire slide at very high resolution to acquire a large number of images for each slide. The images are stored in a database and the virtual slide can be reconstructed using software that puts the single images back together to form a whole.

It was virtual microscopy that led Bacus into the world of tissue microarrays. "Several years ago, there were no good scanning methods for tissue microarrays," says James Bacus, the company's president, "but we had the ability to scan entire slides." Using virtual microscopy as a starting point, Bacus has developed several programs for quantitative, automated TMA analysis including TMAscore and IHCscore.

Several other companies also provide automated TMA analysis solutions. Beecher Instruments has the TMAx software package that relies on morphometric processing algorithms and classification rules to automatically interpret TMAs. And HistoRX in New Haven, Connecticut, offers the AQUA automated quantitative pathology system for biomarker discovery and validation from tissue samples.

With TMAs moving FFPE analysis onto the fast track of high-throughput analysis, all the pieces are falling into place for researchers to begin exploring archival FFPE tissue samples. The coming years could prove very interesting indeed as the molecular secrets of these FFPE samples are finally unlocked. ■

Nathan Blow is the technology editor for *Nature* and *Nature Methods*.